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THE CYTOPATHOLOGY OF VIRUS INFECTION*

Consulting Editor
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INTRODUCTORY REMARKS

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The New York Academy of Sciences has always been interested in information regarding the basic nature and actions of viruses. For this reason it has published such monographs as *Viruses as Causative Agents in Cancer* in 1952, *Virus and Rickettsial Classification and Nomenclature* in 1953, and *Viruses in Search of Disease* in 1957. Certain aspects of viral action have also been presented in the monographs *Subcellular Particles in the Neoplastic Process* and *Hodgkin's Disease* published in 1958.

Despite this interest, comparatively little concentrated attention has been paid until recently to the cytopathology of virus disease. With the introduction of newer techniques such as electron microscopy, cytochemistry, and fluorescent labeling, clarification of many areas of the cytopathology of virus infections has been made possible. The present monograph delineates many of these newer approaches. The information derived, no doubt, will further our knowledge concerning the mechanism of action of viruses on cells, as well as their relation to the neoplastic process. In this connection, important details of the morphologic and metabolic changes induced by a variety of viruses are emphasized.

INTRODUCTION

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Oscar Wilde once said that "Nowadays to be intelligible is to be found out." At the risk of being found out I shall do my best to be intelligible.

Leslie Foulds of the Chester Beatty Research Institute, London, England, tells the following story about cancer research. The street lamps around London end at the outskirts of the suburbs. One night a policeman found a drunk crawling on his hands and knees under the last lamppost in a suburb. In answer to the policeman's query, the man said that he was looking for his key. Being a typical London policeman, the bobby started to look for the key. After about half an hour the policeman, who was sober, began to lose hope and asked if the man was quite sure that he had lost his key near the lamppost. "Not at all," replied the drunk, "I lost it out there," pointing toward the dark, "but this is the only place where there is enough light to look."

When we come to consider the cytopathology of virus infection I think that we confront the same problem. In effect, I have tried to bring together as many lampposts as possible in this monograph. I hope that, now that they are brought together, the combined lighting effect will extend into the regions of darkness.

In some ways I have been compelled to simplify the problem. This monograph omits several important aspects of the virus-cell relationship. There is not enough space to include the significant contributions in the fields of plant and insect viruses and of bacteriophage. With the possible exception of the paper on the Friend virus, the problem of the role of viruses in the production of malignant neoplasia has not been included. I think that we have problems enough, without having to consider the nature of cancer. Furthermore, I feel that it may be helpful at this point to take stock of our present knowledge of what are known conventionally as virus infections, by this I mean those infections characterized by the phenomena ranging from reversible cellular proliferation and hypertrophy without necrosis to those that lead directly to the death of the cell.

There are still many gaps in our understanding of the relationship between the metabolism of a virus and that of the host. What is a Negri body? Antigenic material has been demonstrated in it, but nucleic acids and particles resembling virus have not yet been found. What do we know about the intracellular activity of Newcastle disease virus? Particles go in and particles come out but what happens in between? We know how to prevent poliomyelitis, but we do not know exactly what the virus does, or where it is within the various cells it infects. Viruslike particles have been found in tumors, but nobody knows what they are doing, the viruslike particle is formed after the virus has finished its work. The electron microscopist is filling in many details about the morphologic evolution of the infectious particle. Fundamental virological studies help to elucidate the nature of incomplete, latent, and manifest virus infection and of the genetics of viruses. The formation of

[illegible]

techniques [illegible] It is the main purpose of this monograph to bring these varied disciplines together in the hope of achieving a better synthesis of the knowledge derived from each

[illegible]

is also a useful invention Perhaps it is time to take up arms against a sea of cloudy swelling, hydropic degeneration, and hematoxyphil bodies Our knowledge of cytology and cytochemistry should enable us to be a little more precise Those who say that "necrosis is just necrosis" should be reminded that "death is just death" In fact, they should be made to prove their contention Those [illegible]

proliferation followed by necrosis? What factors determine the outcome of the interaction of a virus and a cell? When we know the answers to some of these questions we shall know something of the teleological significance of changes in the cell I can think of no better system than the study of the virus infected cell to further our understanding of cytopathology

In conclusion, I quote some advice given by the late Welsh poet Dylan Thomas, who said 'The printed page is the place to examine the works of a poem The platform is the place to give the poem the works" I provide the platform and leave it to other contributors to supply "the works"

A COMPARISON OF IN VITRO AND IN VIVO CHARACTERISTICS AS RELATED TO THE PATHOGENESIS OF MEASLES, VARICELLA, AND HERPES ZOSTER*

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With the recent rapid advances in virology resulting from the introduction

Salivary gland virus in tissue culture characteristically produces changes in fibroblastlike cells rather than in epithelial cells,[2] whereas in the human host the reverse is true Many similar examples can be cited

Because of discrepancies such as these, there has been little attempt to correlate cytopathic changes in tissue culture with the pathology and pathogenesis of disease Elucidation of the causes of such differences undoubtedly will lead to a better understanding of virus diseases Consideration of *in vitro* and *in vivo similarities also appears to be of value, for these similarities may, first, pro* vide evidence that a given disease is due to the agent isolated and, second, explain in part the pathology and pathogenesis of the disease in the host In either case, the value of the feature being correlated is somewhat proportional to its degree of specificity for the agent in question The viruses of varicella, zoster and measles each show unique cytopathic changes in tissue culture and, in each instance, there appears to be correlation between these changes and the lesions produced in the human host

Varicella and Herpes Zoster

Most of what is known of the behavior of the agents of varicella and zoster in tissue culture stems from the work of Weller and his co workers[2,7] From this work as well as other studies[8,13] it may be assumed that a single virus is responsible for both clinical diseases Among the evidence that such is the case is the identity of the cytopathic effect of strains of virus isolated from both entities and its unique character

Several types of cells of human, simian, and lapine origin have been shown to be susceptible in tissue culture to this agent and to develop similar changes[6]
... vesicle fluid or other
...ounded
... intra
nuclear inclusions (FIGURE 1) similar to those described by Tyzzer in the skin of patients with varicella[14] Occasional multinucleated cells are seen in such

* The work reported in this paper was supported in part by grants from the National Foundation New York, N Y

foci in tissue culture, as well as in the skin The infected cells show slowly progessive degeneration and may be desquamated or destroyed only after several days The enlargement of the foci proceeds very slowly and after one week or more new or secondary foci appear and expand in turn In cultures made up of spindle-shaped cells with an orderly or organized orientation it is noted that the shape of the degenerative foci corresponds with the prevailing orientation of the cells (FIGURE 2) Such centrifugal spread of the focus is indicative of direct cell to cell transmission and after its initial appearance, is little influenced by the presence of immune serum [8] The secondary foci are explained readily by desquamation of infected cells from a primary focus and reimplantation to serve as niduses for the development of these new foci That

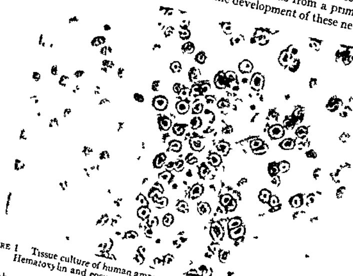


FIGURE 1 Tissue culture of human amnion inoculated with vesicle fluid from patient with varicella Hematoxylin and eosin stain ×467

such is the case is suggested by the work of Weller [8] who was able to recover infectious virus from the supernate of media from cultures showing cytopathic changes in only 1 of 8 attempts although antigen can be demonstrated in tissue culture fluid At best then the liberation of effectively infectious virus from cells is inefficient

The development of the intranuclear inclusions in tissue culture appears to differ somewhat from that occurring in the host in that large basophilic inclusions completely filling the nucleus are not seen Rather inclusions appear to begin as one or more small amphophilic or eosinophilic bodies that gradually enlarge and are associated with increasingly prominent halos and chromatin margination Such nuclear changes may occur prior to the development of changes detectable in unstained cells

Analogies to the characteristics seen in tissue culture are readily apparent in the pathology of human disease produced by this agent [8, 14, 15] In the course

of the viremia of varicella focal lesions develop in the skin and at least in fatal

as does a small group of overlying epidermal cell In time the epidermal involvement becomes vesicular and expands (FIGURE 3) Necrosis is not as prominent in the skin lesions of chicken pox as it is in those of herpes simplex and vaccinia

The lesions of varicella seen in almost all organs are likewise strikingly focal in character In such lesions many different types of cells may show inclusions including the vascular endothelium Hemorrhage is frequently present in and about these foci of necrosis as further evidence of endothelial damage In the



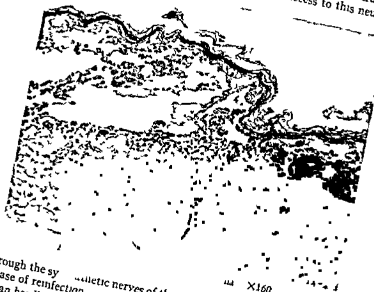
FIGURE 2 Tissue culture of human embryonic skin muscle inoculated with strain of varicella Unstained ×39

patient necrosis undoubtedly of viral etiology is not always accompanied by the presence of inclusions whereas in tissue culture all the degenerating cells contain these bodies This is particularly the case in the liver in which inclusions are demonstrated with difficulty if at all Thus in the host the lesions somewhat resemble an embolic phenomenon with early involvement of the endothelium and local spread of the infection from cell to cell

The comparison of the *in vitro* and *in vivo* characteristics of infection in varicella would appear complete if it could be shown that the virus is transported from the initial site of infection to the skin and other organs within one of the formed elements of the blood such as the lymphocytes We have seen on one occasion inclusions in the thymus in cells that were thought to be lymphocytes [15] Such a carrier cell might explain in part the severity of the infections in patients with leukemia

In keeping with the monistic theory[10] of the etiology of varicella zoster the clinical and pathological differences in the two diseases may be explained with

out invoking differences in tropism of virus strains, particularly in light of the *in vitro* characteristics of the virus Herpes zoster typically occurs in patients who have had a previous varicella infection and who would then be expected to have a humoral immunity tending to prevent hematogenous dissemination *In shingles* there is clinical and pathological evidence that spread of the virus occurs along nerves Inclusions have been demonstrated in ganglion and satellite cells of the dorsal root ganglia corresponding to the dermatome involved, and they have also been seen[15, 16] in the sympathetic nerves (FIGURE 4) The site and mode of access to this neural pathway are not known, although it appears that herpes zoster may either represent activation of latent virus remaining from a previous varicella infection or may gain access to this neural path



ua X160

way through the sy ..thetic nerves of the respiratory or gastrointestinal tracts in the case of reinfection

One can hardly imagine properties more ideally suited to the pathogenesis of herpes zoster than those observed *in vitro* In addition to the inclusion bodies and the relatively slowly progressive changes leading to necrosis one might cite the focal nature of the lesions in the tissue culture the spread of the infection by cell to-cell contact and the lack of influence of antibodies in the tissue culture media on this spread Although there must be abundant opportunities for the virus to gain access to the blood in the various lesions of herpes zoster particularly in the skin in which inclusions may be seen in the capillary endothelium secondary hematogenic involvement of the skin usually does not occur probably because of the presence of humoral antibodies Such would not be expected to prevent the development of the zoster lesion itself if the spread is actually along cellular neural pathways.

homogeneous amphophilic material without a surrounding halo as has been illustrated by Bonenfant[22] Although there are other possible mechanisms whereby such cells might originate, their striking similarity to the changes observed in tissue culture is strong evidence that the method of production of giant cells is similar *in vivo* and *in vitro*

Although epithelial giant cells in the respiratory tract have been noted in pathological descriptions[23] they did not receive much attention until their appearance in smears of nasal secretions was found to be of value as a diag nostic tool by Tompkins *et al*[24] We have also seen such epithelial giant cells in the mucous glands of the trachea in a typical proved case of measles dying shortly after the appearance[25] of the rash (FIGURE 8) Their similarity to the cytopathogenicity of measles virus in tissue culture is even more striking than

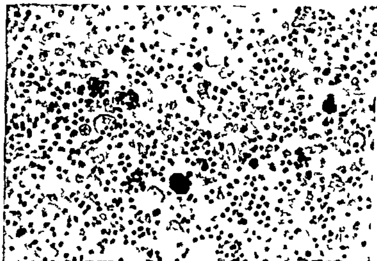


FIGURE 7 Thymus from case of measles showing development of Warthin Finkeldey giant cells X564

that of the Warthin Finkeldey cells In these multinucleated cells could be found numerous intranuclear inclusions although cytoplasmic inclusions were not evident Although there was an interstitial bronchopneumonia that was probably due to measles virus these giant cells were not seen in the lung in this case

Another lesion that may be produced by infection with the virus of measles
was first described by F
giant cell pneumonia
fection is more chronic
tures are quite striking and are those of a subacute or chronic interstitial bronchopneumonia with metaplasia of bronchial and bronchiolar epithelium and alveolar lining cells (FIGURE 9) From fusion of such cells large multinucleated giant cells containing intranuclear and sometimes also intracytoplasmic inclusions can be seen (FIGURES 10 11) Such cases due to measles virus can usu

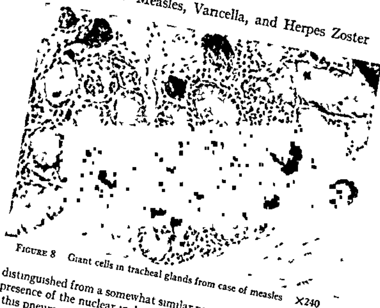


FIGURE 8 Giant cells in tracheal glands from case of measles ×240

ally be distinguished from a somewhat similar pneumonia of unknown etiology[28] by the presence of the nuclear inclusions

That this pneumonia in children is due to infection with the virus of measles s supported by clinical association with measles infection and the morphologic imilarity to the lesions of typical measles infection and the … logenicity

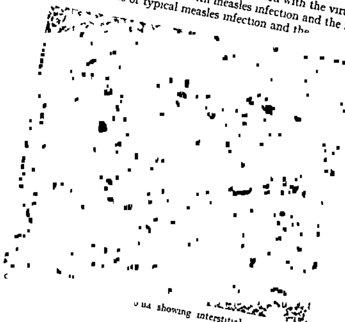


FIGURE … showing interstitial infiltrate and epithelial metaplasia

16 CHEATHAM, W J 1953 The relationship of heretofore unreported lesions to patho-

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DOUBLE INFECTION OF CELLS IN CULTURE WITH MEASLES AND POLIOMYELITIS VIRUSES*

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When a susceptible cell is exposed to two different viruses, two types of reaction may ensue: one virus may interfere with the penetration and/or the multiplication of the second, or double infection of the cell with both viruses may occur. On the basis of morphologic evidence it has been reported[1, 2] that dual infection could be observed when one virus induced intranuclear and the other cytoplasmic inclusions, but not when the inclusions induced by the two viruses had the same intracellular localization. Double infection of a cell has been reported by Anderson[1] with fowlpox and herpes simplex, with fowlpox and laryngotracheitis, or with herpes simplex and rabies viruses. Similar findings were reported by Syverton and Berry[2] for vaccinia and either herpes simplex or B virus. Double infection of a cell with vaccinia and fowlpox, two viruses inducing the formation of inclusion bodies with the same intracellular localization, could not be observed microscopically.

In the course of studies with poliovirus in monkey kidney (MK) cultures, it was observed that cultures spontaneously infected with the monkey intranuclear inclusion agent of Ruckle[3] were apparently fully susceptible to poliovirus. Poliovirus induced inclusions were seen in the nuclei that already contained inclusions induced presumably by the monkey agent. Since poliovirus multiplied in cells apparently infected with the Ruckle agent, as observed cytologically, experiments were carried out to determine whether normal cells would support the growth of both agents at the same time. The present report describes experiments on the double infection of MK cells with measles virus (which is apparently identical with the monkey intranuclear inclusion agent[4]) and with poliovirus. In MK cells, measles virus infection is characterized by large intranuclear and cytoplasmic inclusion bodies and by the appearance of multinucleated cells,[4] whereas poliovirus induces the formation of eosinophilic cytoplasmic masses and small intranuclear inclusion bodies.[5] These cellular alterations are quite distinct in their morphologic characteristics, and changes induced by each virus can be distinguished readily.

Experimental Results

After poliovirus infection of MK cells, newly formed cell associated virus appears as soon as 3 hours after inoculation.[6] The growth of measles virus, on the other hand, is far slower, and cell associated virus appears approximately 18 hours after inoculation.[7] In addition, presumably because of the low infective titers of measles virus and its instability at 37° C, this agent does not

* The work reported in this paper was supported in part by a grant from The American Cancer Society, New York, N.Y.

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produce generalized cytopathic degeneration of the cells during the first growth cycle

The experiments therefore, were designed to take the different growth rate of both viruses into account and the following experimental procedure was adopted primary cultures of MK cells grown in Hank s saline containing 2 per cent calf serum, were inoculated with measles virus and 5 to 7 days later when the monolayers showed generalized measles induced cytopathic changes the cultures were inoculated with poliovirus The fluids and cells from these measles infected cultures were assayed at various times after inoculation with poliovirus until the monolayer was completely degenerated The cultures were also examined cytologically Parallel cultures inoculated with either poliovirus or measles virus alone were harvested at the same time for comparison with the doubly infected ones At each selected interval 6 samples from each series were harvested 3 of the samples were frozen at −70° C for virus titra

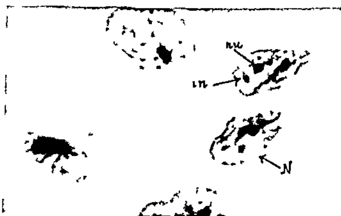


FIGURE 1 Cells from a monkey k dney culture inoculated with pol ovirus Early stage of cytopath c legenerat on The nucleus at the top app ears normal N nucleus *nu* nucleolus *in* *intranuclear inclusion* *Approximately ×1200*

tion the other 3 were fixed in Zenker s fluid and were stained with hematoxylin

d by measles and by
[6] In the early stages
intranuclear inclusion
pear darker wrinkling

of the nucleus occurs and most of the chrom tin r et ork disappears[6] (FIGURE 1) Later a large eosinophilic paranuclear mass appears in the cytoplasm and the cell detaches from the glass and becomes rounded (FIGURE 2) Measles virus induces the formation of multinucleated giant cells with acidophilic intra nuclear and cytoplasmic inclusions surrounded by a clear halo[4] (FIGURE 3) These intranuclear inclusions differ from the ones induced by poliovirus in that they are much lighter in color, the chromatin network disappears only

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Cultures previously infected with measles virus are fully susceptible to poliovirus, as determined by observation of the cytopathic changes induced. When



FIGURE 2. Cells from a culture similar to that shown in FIGURE 1 but in a more advanced stage of cytopathic degeneration. N, nucleus; nu, nucleolus; in, intranuclear inclusion; sc, eosinophilic cytoplasmic mass. Approximately ×1500.

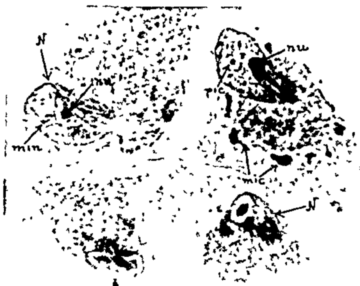


FIGURE 3. Multinucleated cell from a monkey kidney culture inoculated with measles virus. N, nucleus; nu, nucleolus; in, intranuclear inclusions; ic, cytoplasmic inclusion. Approximately ×1200.

such doubly infected cultures are fixed and stained, changes typical of both measles and of poliovirus-induced degeneration frequently can be seen together in many mononucleated and multinucleated cells. Many cells that show wrinkled nuclei with darkly outlined membranes and eosinophilic cytoplasmic masses contain a large light colored intranuclear inclusion typical of measles infection (FIGURES 4 and 5). A number of these cells contain, in addition to the measles-induced intranuclear inclusion, one or several smaller and darker

intranuclear inclusion bodies similar to those seen in polio infected MK cultures, which have never been seen after infection with measles virus alone [illegible]

In addition to the morphologic changes just described, it is not unusual to find in multinucleated cells that only some nuclei in a syncytium show abnormal changes or that these changes are shown to varying degrees (FIGURES 7 and 8) When such a cell contains an eosinophilic cytoplasmic mass, the



[illegible] 1 [illegible] infected with measles and with [illegible]

the result of superimposed cells

Virus yields from multicellular cultures Several experiments were performed using the procedure outlined above One of these experiments will be described in detail

Thirty six cultures were inoculated with approximately 10^4 tissue culture half dose (TCD_{50}) of measles virus, Edmonston strain Another 36 cultures



FIGURE 6 ... poliovirus ... which the ... cytoplasm ... clusion. Approximately ×1200





FIGURE 8 Cell from the same culture shown n FIGURE 7 Th s multinucleated cell con tains both normal and wrinkled nucle Two of the wrinkled nucle contain large measles-l ke inclus ons Approximately ×540

from the same lot were set aside and handled in the same manner, but were not inoculated with virus The virus inoculated into the first set of tubes was allowed to adsorb to the cells for 4 hours at 37° C, when unadsorbed virus was removed and fresh medium was added On the fifth day, when generalized cytopathic changes were seen and many cells had already become detached from the glass, 18 of the measles-infected cultures were inoculated with about 120 plaque forming units (PFU) of poliovirus, Mahoney strain In the remaining 18 cultures the nutrient was changed and the tubes were reincubated At this time 18 cultures not previously infected with measles were also inoculated with poliovirus and handled in the same manner as the doubly infected

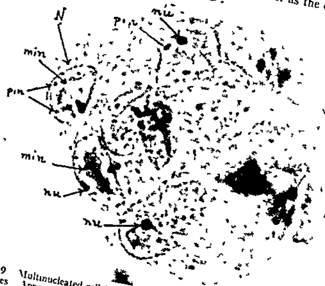


FIGURE 9 Multinucleated cell from the same doubly infected culture shown in the preceding figures Approximately ×1200

ones Poliovirus was allowed to adsorb on the cells for 1½ hours, the cultures were washed in saline, fresh medium was added, and the cultures were then reincubated at 37° C Six cultures from each series 3 for cytological observation and 3 for virus titration, were harvested at 20, 48, and 72 hours after poliovirus inoculation Titrations of measles virus were carried out in cultures of HEp-2 cells These cells were used instead of MK because sometimes viruslike agents which appear spontaneously in MK cultures,[2,4] cause cytopathic changes of the cells that cannot be distinguished from those indu casl virus Titrations of poliovirus were done on MK cell t of Dulbecco and Vogt[3], as modified by Hsiung and 7 fluid from doubly infected cultures, which and poliovirus the presence of measles virus did not poliovirus on MK cells However yed the measles virus, the poliovirus pr

pathic effect of measles virus could be detected Because of the rapid cell destruction caused by poliovirus, it was necessary to carry out titrations of measles virus from doubly infected cultures in the presence of type specific poliovirus antiserum The samples to be assayed were mixed with equal amounts of antiserum, which neutralized at least 10^4 TCD_{50} of poliovirus, and were kept at room temperature for one half hour before being inoculated into culture tubes Since normal monkey serum often contains antibodies to mea

TABLE 1

POLIOVIRUS YIELDS OF CULTURES PREVIOUSLY INFECTED WITH MEASLES VIRUS*

Hours after poliovirus inoculation	Control cultures poliovirus alone (log PFU/culture)	Doubly infected cultures (log PFU/culture)
20	5 6	5 7
	5 8	6 0
	5 8	6 1
48	7 7	7 7
	7 8	7 7
	7 8	7 8
72	7 4	Cell sheet completely destroyed at 50 hours
	7 8	
	7 9	

* Poliovirus inoculum 1 2 × 10^2 PFU, Type I, Mahoney

TABLE 2

MEASLES VIRUS ASSAYS FROM CULTURES BEFORE AND AFTER INFECTION WITH POLIOVIRUS*

Hours after poliovirus inoculation	Control cultures measles alone (log TCD_{50}/culture)	Doubly infected cultures (log TCD_{50}/culture)
0	3 0	—
	3 0	—
	3 7	—
20	4 5	3 5
	3 7	3 0
	3 7	3 0
48	4 0	3 7
	3 7	2 7
72	4 0	Cell sheet completely destroyed at 50 hours
	3 7	

* Inoculated with 1 2 × 10^2 PFU of Type I, Mahoney poliovirus 5 days after measles virus infection

sles virus,[10] the polio antisera used were made in rabbits and did not inhibit measles virus growth

The poliovirus yields from the doubly infected cultures were as high as those from cultures inoculated with poliovirus alone (TABLE 1), although the number of cells present in the cultures at the time of poliovirus inoculation was lower because of the previous cytopathic effect of measles virus In contrast, the measles virus yields were about ten times lower in the doubly infected cultures (TABLE 2) Destruction of the cell sheet by poliovirus occurred about 24 hours sooner in the doubly infected cultures than in parallel cultures infected with poliovirus alone

The results of the virus assays support the morphologic observations and they suggest strongly that poliovirus can multiply in a cell already infected with measles virus. However, cytological examination of parallel cultures infected only with measles virus and harvested at the time of poliovirus inoculation showed that about 5 per cent of the cells did not show measles induced changes as determined by morphologic criteria. The poliovirus produced by these nonmeasles infected cells could represent a large proportion of the total virus produced by the doubly infected cultures. An attempt was made to evaluate the production of poliovirus by doubly infected cells in a more precise manner by comparing the virus yields of single cells from doubly infected cultures and from cultures infected with poliovirus alone isolated in microdrops under oil according to the technique of Lwoff and Dulbecco 11.

Experiments on isolated single cells. This work was done with monolayer cultures of MK cells that had been inoculated with measles virus and that showed generalized cytopathic changes with areas of cell destruction. At this time the cultures were inoculated with poliovirus calculated to give an input multiplicity of at least 5 PFU/cell. After an adsorption period of 1½ hours the monolayers were washed and treated with disodium ethylenediamine tetraacetate (Versene) 0.02 per cent to obtain a suitable cell suspension. The cell suspension was then washed twice and resuspended in nutrient medium (Hank's saline with 0.5 per cent lactalbumin hydrolyzate and 2 per cent calf serum). Single cells were isolated in microdrops under Bayol in Petri dishes by freehand manipulation under a dissecting microscope at a magnification of 150. The microdrops then were checked carefully under the microscope to be sure that they contained only one cell. The diameter of the cells was measured by means of an ocular micrometer and the dishes were reincubated at 37° C in a humidified atmosphere containing 5 per cent CO_2 for 7 more hours. To harvest the cultures the Petri dishes were removed from the incubator and the microdrops were frozen at −20° C *in situ* in the oil. The contents of the dish were thawed just prior to titration and each microdrop was diluted in 1.0 or 2.0 ml of nutrient medium.

The cells were measured under the microscope with an ocular micrometer after isolation in microdrops and classified according to their morphologic characteristics into round cells of normal size, giant round cells and giant flat cells. The round cells of normal size had a diameter ranging from 8 to 64 μ, most of them measuring from 16 to 40 μ. Cells of this size were found in uninoculated cultures and in cultures infected with poliovirus as well as in measles infected cultures. The other two cell types were observed only in suspensions prepared from measles infected cultures: the giant round cells were always multinucleated and often measured several hundred microns in diameter (the few that measured less than 100 μ were not included in the data). The flat giant cells were relatively infrequent; they were multinucleated measles infected cells that had failed to round up under the action of Versene, did not attach to the glass, and presumably were dead.

TABLE 3 shows the combined results of 8 such experiments performed on isolated cells infected with poliovirus alone and of 16 experiments performed on doubly infected cells. The results of the virus yields from mononucleated cells of normal size are similar in the measles and in the nonmeasles infected

cultures, the difference between them being within the range of variation found in different experiments The methods employed have not enabled us to detect measles virus production at the single cell level, which would have made it possible to determine whether a particular single cell was doubly infected The yields from the multinucleated cells are particularly interesting and distinctly higher these giant cells were always multinucleated, they were never seen in cultures infected only with poliovirus, and when stained they showed the typical measles induced cytopathic changes These doubly infected giant round cells not only produced poliovirus, but their yields of poliovirus were much higher than those obtained from small mononucleated cells Some of these cells have produced more than 4000 PFU Two different sets of control experiments support the view that the large amount of poliovirus associated with these cells was actually produced by them and was not merely the result of adsorption of the inoculum to the large surface of these cells Giant cells isolated in microdrops and frozen immediately thereafter, without allowing

TABLE 3
POLIOVIRUS YIELDS FROM SINGLE CELLS

PFU per microdrop	Control drops medium No cells	Round cells of normal size		Giant round cells	
		Polio alone	Doubly infected	Polio alone	Doubly infected
0	65	100	122	—	41
1–10	3	16	43	—	11
11–100	5	37	24	—	16
101–200	0	18	3	—	4
201–1000	0	7	9	—	10
1000+	0	0	0	—	10
Total cells	73	175	201	—	92

time for virus multiplication, seldom contained poliovirus When they did contain poliovirus, there was always less than 50 PFU/cell Microdrops containing large multinucleated flat cells that were also found in the suspension and were presumably dead, since they failed to round up under the action of Versene and did not attach to the glass, were also studied These cells were isolated, reincubated, and assayed for poliovirus Thirty one giant flat cells were tested 14 were negative, 15 yielded less than 10 PFU, and 2 yielded between 10 and 50 PFU

All assays for measles virus produced by single cells were negative, not only in doubly infected cultures, but also in cultures infected only with measles virus This was true even in cases when it was shown that measles virus was being produced by the monolayer before being dispersed into single cells In 6 separate experiments, 116 mononucleated and multinucleated cells from inoculated cultures were tested for measles virus These cells were isolated from cultures that had been infected with measles virus from 5 to 8 days previously and showed moderate to advanced cytopathic degeneration The cells were reincubated in microdrops after isolation for periods ranging from 9 to 36 hours, but no virus could be detected Fluid from the monolayer cultures used in

these experiments was harvested just before isolation of the single cells and contained significant amounts of measles virus which, in view of the rapid inactivation of the virus at 37° C, must have been released a short time previously

Discussion

The present data show that it is possible to infect a cell with measles and poliovirus, each of which produces alterations and inclusion bodies in the nucleus and in the cytoplasm. Previously double infection of a cell had been demonstrated only when the inclusion bodies produced by both viruses had different intracellular localizations. The morphologic changes induced by measles and by poliovirus are quite characteristic and can be readily distinguished. Although it is quite possible that both kinds of virus particles are actually present in the same portion of the cell, our observations do not necessarily prove this. Specific virus-induced inclusion bodies are not always composed of aggregates of virus particles. In the case of measles and of poliovirus, the electron microscopic evidence available would suggest instead that they are not. Extensive electron microscopic observations of cells infected with these viruses have been made and so far no virus-like particles have been found. Furthermore the measles-induced intranuclear inclusions correspond to areas of low electron density where the chromatin network has disappeared and particles could not be seen.

These morphologic observations, strongly suggestive of double infection, are supported by the results of the virus assays. Study of the virus yield from doubly infected monolayer cultures shows that previous infection with measles virus does not interfere with poliovirus multiplication. Similarly, measles virus production continues for some time after infection with poliovirus, although the virus titers are significantly reduced. It is not possible to determine whether this reduction in titer is due to a decrease in the amount of measles virus produced by the doubly infected cells or to complete cessation of measles virus production of those cells superinfected with poliovirus.

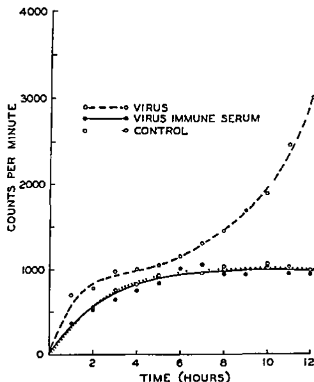
Evidence of double infection at the single cell level was obtained in the case [illegible]

be explained by the large volume of these syncytial masses. However at present it is not possible to determine whether measles virus plays any role in enhancing or depressing the multiplication of poliovirus in the same cell.

Acknowledgements

I thank Joseph L. Melnick and Frances L. Black for their advice and suggestions in the course of this work. I also thank Isabel Morgan M[illegible] for supplying the polio antisera.

This transudation phenomenon closely parallels the release of virus and the progressive loss of cytoplasmic area seen in time lapse cinemaphotography (unpublished results, W W Ackermann and H Kurtz) It is probably related to the mechanism of viral release and due to specific virus action since the extent of the process in any culture is dependent upon the concentration of the viral



[illegible]

inoculum and can be prevented by prior incubation of the virus with viral specific immune monkey serum

These observations tell us little of the biochemical alterations leading to this selective loss of cellular integrity, but they do determine the maximum interval after infection that can be studied most profitably This period comprises the first seven hours when nearly all the newly formed virus is intracellular and the essential integrity of the cell is intact

Results that are more easily subject to interpretation are obtained by following the incorporation of P^{32}, which is introduced after infection

The object of the next experiment is to follow the incorporation into various tissue components of the labeled phosphate that was introduced at various times after infection. The essential part of the observation has already been recorded in the literature (Maassab *et al.*, 1957).

In a typical experiment, a series of cultures was exposed to a large inoculum of poliovirus. After 1 hour of incubation at 37° C., the cultures were washed free of the residual inoculum and incubated further. At various times between 1 and 7 hours after initiation of infection, each culture was exposed to P^{32} in the form of inorganic phosphate. One half hour after this addition the experiment was terminated. The cells were fractionated into cytoplasm and nuclei. Both morphologic fractions were subjected to chemical analysis. In addition, a sample of the cytoplasmic fraction was taken for virus assay. Thus the accumulated increase of nucleic acids, the rate of incorporation of P^{32} over short time intervals, and the appearance of the virus were followed.

The Nucleic Acid Composition of Normal HeLa Cells and Their Incorporation of P^{32}

When ordinary HeLa cells were fractionated into cytoplasm and nucleus and analyzed, the total ribonucleic acid (RNA) was found to be 1.5 to 2.5 times greater than the desoxyribonucleic acid (DNA). The amounts of nuclear RNA and cytoplasmic RNA were quite similar. The uptake of P^{32} into various nucleic acid fractions follows the pattern of cells from other species described previously (Marshak, 1948; Smellie *et al.*, 1953). The highest rate of incorporation is seen in the nuclear RNA, followed by the cytoplasmic RNA, and DNA. The amount of P^{32} incorporated into the total RNA is 17.8 times greater than that in DNA. This phenomenon generally has been found for resting cells (Smellie *et al.*, 1953). In the experimental period studied, from 0 to 7 hours, it was found that the cell number, composition with regard to DNA and RNA, and the pattern of incorporation of the isotope did not vary significantly.

Incorporation of P^{32} into the Nucleic Acids During the Infectious Cycle

In contrast to the constant activities of the normal cell, striking changes in uptake of P^{32} occur after infection. The data in FIGURE 2 illustrate the rate of uptake of P^{32} during various intervals of the infectious cycle. Since the pattern of incorporation of the isotope by the uninfected controls did not vary significantly throughout the period studied, the values obtained for the incorporation of P^{32} are recorded as percentage change relative to the control. The enhanced incorporation of P^{32} by the 3 nucleic acid fractions is detectable by the first hour after infection. It is sustained in the DNA for 2 hours, after which it steadily declines but more abruptly at the fourth hour of infection. In contrast, the cytoplasmic RNA increases in activity until the sixth hour, after which it also decreases. Becker *et al.* (1958) have observed also increased incorporation of P^{32} into human amnion cells following infection. Similarly, Miroff *et al.* (1957) found an increased incorporation of P^{32} into the total nucleic acids of HeLa cells during the first 2 hours of infection with poliovirus.

Changes in Nucleic Acids, Protein and Virus Following Infection

Cultures of HeLa cells were analyzed for changes in the nucleic acid phosphorus at various times after infection. The nucleic acids of the nuclear fraction show a slight rise for a period of 2 hours after infection and remain constant thereafter. The increase in cytoplasmic RNA P starts by the first hour and is progressive until the sixth hour, at which time the RNA P may have increased 230 per cent. Each fraction of the cytoplasm examined during the period of infection studied also shows increases in protein. The amount of increase in protein at 7 hours represents nearly a doubling of the cytoplasmic

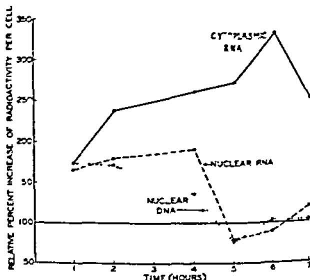


FIGURE 2 Rate of P^{32} incorporated per half hour into the 3 nucleic acid fractions. Cells were exposed to an undiluted inoculum containing a total of 1×10^9 PFU of virus for a period of 1 hour. Cultures were washed 3 times with Hank's balanced salt solution, supplied with fresh medium and incubated at 37° C. The additions of P^{32} contained a total of 125 μc per culture. The amount of P^{32} incorporated is expressed as the percentage of change related to the control.

material. The first increase in viral activity in the cell was not detected until the fourth hour; extracellular virus starts to appear at about the sixth hour. It should be noted that at the seventh hour, when 90 per cent of the total viral yield has been formed, only 1 per cent of the new virus is in the extracellular state. These data clearly indicate an intracellular phase in the development of poliovirus, as has been reported by other investigators (Roizman *et al.*, 1958; Darnell, 1958).

The increases in nucleic acids and protein described here are huge in proportion to the size of the cell. The significance of the changes can be deduced f[illegible]

[illegible] ess of what would be incremental RNA and

protein were all virus, the yield would be 10^8 particles/cell, and the protein would correspond to 10^7/cell. However, the yield of virus seldom exceeds 1000 plaque forming units (PFU)/HeLa cell. According to Schwerdt and Fogh (1957), the number of characteristic particles in a preparation that are countable with the electron microscope is not less than 30 PFU/cell under the best plating conditions. This would set a lower limit of 30 000 particles/HeLa cell. The increase in RNA and protein in the cytoplasm actually found at the sixth hour of infection is of a different order of magnitude than the increase in viral activity.

Second, the composition of the cytoplasmic RNA is not altered in the direction expected by the accumulation of additional RNA of the virus type. The RNA of the cytoplasm obtained after 6 hours from both infected and normal HeLa cells was hydrolyzed to a mixture of nucleotides by the method of Volkin and Carter (1951) and chromatographically separated as described by Hurlbert *et al.* (1954). The chromatographic fractionation of the hydrolyzate

TABLE 1

AMOUNT OF EACH NUCLEOTIDE IN CYTOPLASMIC RNA (cRNA) OF NORMAL AND INFECTED HeLa CELLS

Nucleotide	μM of nucleotide × 10[illegible]/cell		
	Normal	Infected	Nucleotide*
Cytidylic acid	11.26 ± 3.4	28.75 ± 8.6	17.49
Adenylic acid	7.4 ± 1.9	19.05 ± 6.1	11.65
Guanylic acid	16.05 ± 3.1	38.49 ± 8.8	22.44
Uridylic acid	8.41 ± 1.7	21.03 ± 7.54	12.62

Samples for analysis were obtained from HeLa cells 6 hours after initiation of infection with poliovirus and from normal HeLa cells treated in the same manner without exposure to virus. Values recorded here are averages of data from 5 experiments.

* The difference between normal and infected cells in the amount of each nucleotide in the cRNA.

of the nucleic acids yielded the usual 4 nucleotides. The number of micromoles per cell of each nucleotide was determined. The results are presented in TABLE 1. There is approximately 150 per cent more of each nucleotide in the cytoplasmic RNA of the infected as compared to the normal cell. The nucleotide composition of the RNA isolated from the cytoplasm of normal HeLa cells is very similar to that reported for RNA isolated from other mammalian tissues in that it is rich in guanylic and cytidylic acids and poor in adenylic and uridylic acids. The RNA of the cytoplasm of infected cells was found to have a nucleotide composition that does not differ significantly from that of normal cells. However values obtained by Schwerdt (1957) for the nucleotides of RNA from poliovirus Mahoney strain are quite different in composition from those of normal cells. The values are lower especially for cytidylic and guanylic acids. Thus while viral RNA must be synthesized in the infected cell the major portion of the newly formed RNA induced by virus infection appears not to be of the viral type but resembles more closely that of the ordinary cell cytoplasm.

Third the distribution of the incremental materials among the various fractions does not correspond to the distribution of virus. Furthermore, in two of

the fractions the incremental protein and RNA are not present in the same proportions as in the virus

The cytoplasmic RNA is a composite of several species of RNA (Smellie, 1955) associated with various subcellular components By differential centrifugation the subcellular elements may be fractionated and thus indicate where the incremental RNA exists in the cytoplasm of infected cells At the sixth hour of infection the cytoplasm of normal and infected HeLa cells was fractionated by differential centrifugation, following the method of Hogeboom and Schneider (1955) into 3 fractions Fraction I, the sediment after 6600 g for 20 min , Fraction II the sediment after 41000 g for 1 hour, and Fraction III, the supernatant fluid above Fraction II Each of these fractions was analyzed for RNA P protein and virus activity, and the rate of incorporation of P^{32} into RNA

TABLE 2
DISTRIBUTION OF PROTEIN N AND RNA P IN THE CYTOPLASMIC COMPONENTS OF NORMAL AND INFECTED HELA CELLS

Cytoplasmic fraction	Normal			Infected			
	RNA P		mg N × 10^{-10}	RNA P		mg N × 10^{-10}	Virus PFU
	Counts* × 10^{-4}	mg × 10^{-9}		Counts × 10^{-4}	mg × 10^{-9}		
I	1 45	1 95	59 74	2 73	3 21	91 52	15 5
II	1 25	4 55	54 37	2 88	13 26	73 05	313 5
III	4 45	20 38	248 19	6 54	25 51	379 87	54 5

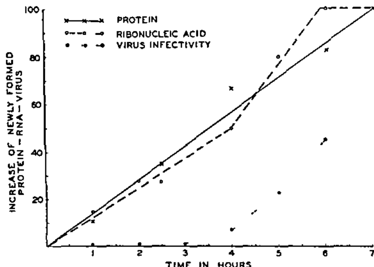
Cytoplasm for ultracentrifugal fractionation was obtained from HeLa cells 6 hours after

The data are presented in TABLE 2 In the normal cell, the distribution of the cytoplasmic RNA among the 3 fractions is 7, 18, and 75 per cent, respectively P^{32} was incorporated into the RNA of each fraction of the uninfected cell The specific rates of incorporation of the isotope vary, Fraction III containing 75 per cent of the cytoplasmic RNA is the least active After infection there is an increase in the rates of incorporation of the isotope in all of the fractions Fraction II showing the greatest increase (130 4 per cent) The distribution of total RNA P follows the same pattern seen with the radioactivity data After

fractions show an increase in protein content The largest accumulation is in Fraction III, but the percentages in fractions I and III are the same and are greater than in Fraction II The virus activity is found predominantly in Fraction II

There is a characteristic value for the ratio of protein N to RNA P for each of the components of the normal and infected cell cytoplasm and for the purified virus as computed by Schwerdt (1957) A comparison of these values de-

termines whether the newly formed materials resemble the composition of the normal component with which it is associated or that of the virus or whether the materials are anomalous Only in Fraction II does the incremental material resemble that of the virus The incremental materials of Fraction I more closely resemble the normal components than the virus Fraction III of the infected cells is seen to contain an increment that is relatively rich in protein as compared to the normal component From these considerations we conclude that the accumulated protein and RNA do not constitute poliovirus and at least for RNA do represent material of the virual type (Ackermann *et al* 1958)



[illegible]
following infection
Key X——X——X protein of Fraction III of cytoplasm O – O – O ribonucleic acid of total cytoplasm ● ● ● virus infectivity of total cytoplasm

Kinetics of Synthesis of RNA Protein and Virus

The preceding sections describe the biochemical situation in the cell at the sixth hour of infection The following experiment is concerned with the sequence of steps in time by which the situation developed Replicate cultures of cells were analyzed at various times in the interval from the initiation of infection to the seventh hour In order that the characteristics of development of several materials that differ in absolute amount may be compared the incremental amount of each at the seventh hour was assigned the value of 100 and the amounts at other times some proportion of 100 In this form data concerning the newly formed protein of Fraction III of the cytoplasm the total cytoplasmic RNA and virus infectivity are plotted in FIGURE 3 Th

synthesis of protein proceeds at a constant linear rate from the first hour until the seventh. The synthesis of RNA also begins close upon the initiation of infection and appears to be linear; the rate in the first 4 hours closely parallels that of the protein synthesis. The rate between the fourth and sixth hours proceeds at a further increased rate, and the process stops at the sixth hour. It appears that 50 per cent of the protein and RNA are formed before the appearance of any virus activity. A further 50 per cent of the virus appears after synthesis of RNA stops.

Currently, a similar study is under way. The amounts of RNA and virus were determined in HeLa cells at various times during a single sequence of infection with hemadsorption Type I virus. Some of the preliminary observations are presented in TABLE 3. From these data the following interpretations are drawn concerning the biosynthetic activities of the infected cell.

TABLE 3
PHOSPHORUS DISTRIBUTION OF HELA CELLS INFECTED WITH HEMADSORPTION VIRUS TYPE I

Hours after infection	mg × 10[illegible] per cell*				Virus titer	
	CRNA P		Total NA P of nuclei		TCID₅₀ ml	
	C	I	C	I	Extra	Intra
2	21 59	29 9	26 90	30 90	2 5	3 5
6	—	28 33	—	32 10	2 7	3 3
12	26 13	52 45	34 2	41 50	4 3	7 0
18	—	50 22	—	49 9	5 0	7 9
24	25 99	35 2	31 0	33 0	6 8	7 7

* [illegible]

cells were taken for virus assay

The virus induces nucleic acid synthesis in both of the major morphologic fractions of the cell (nucleus and cytoplasm). There is an increase in the cytoplasmic RNA fraction even at the second hour and a marked accumulation of RNA P between the sixth and twelfth hours. After reaching its maximum value at the twelfth hour of infection, the level of RNA P of the cytoplasm declines and the release of the virus from the cell is apparent. The nucleus, on the other hand, shows a sustained accumulation of nucleic acid for a period of at least 18 hours and then declines.

Associated studies of viral activity relate the appearance of new virus and nucleic acid synthesis. Viral increase parallels more closely the induced changes of the nucleic acid of the nuclear fraction. There is also an intracellular phase of viral development as found previously for poliovirus. Thus the virus seems to induce an increase of the nucleic acid of the nucleus as well as the cytoplasm of the infected cell, in contrast to poliovirus, where the incremental material is

located primarily in the cytoplasm A further characterization of this incremental material is in progress

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RELATIONSHIP OF VIRUS MULTIPLICATION TO THE DEVELOPMENT OF MORPHOLOGICAL AND BIOCHEMICAL CHANGES IN THE CELL*

Charles C Randall W [illegible] M [illegible] ld Glenn A Gentry
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A number of workers have vari [illegible] histochemical and cytochemical techniques with the objecti [illegible] r ing changes in the infected cell The literature cited has been [illegible] arbitrarily to mammalian viruses Hyden[1] used ultraviolet microscopy [illegible] nstrate that a number of virus infections resulted in increased de [illegible] acid (DNA) or ribonucleic acid (RNA) Scott and his collegu [illegible] ve made a classic study of herpes simplex and have correlated morp [illegible] hanges with the growth cycle, and have also made a study of the Feulgen reaction and observed that positive inclusions become negative with t me thi was confirmed by Wolman and Behar[6] Lépine and Sautter[7] failed t find Feulgen positive material in the inclusions of herpes simplex Cowdry [illegible] demonstrated inclusions that stained equivocally with the same technique Blank[8] has observed that the nuclei of human wart lesions and the intracytoplasmic inclusions of molluscum conta [illegible]

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Studies with Equine Abortion Virus (EAV)

An attempt has been made to relate morphologic changes as depicted by hematoxylin and eosin (H and E) and Feulgen stains with the growth cycle[16] and certain chemical changes in cell nuclei.[17]

Three week-old Syrian hamsters are extremely susceptible to infection with EAV. Animals inoculated intraperitoneally with 1 ml each of a 1:5 dilution of freshly ground infected liver containing approximately 10^{9} LD_{50} usually succumb with a rapidly lethal hepatitis within 18 to 24 hours. In a sequential study infected animals were compared with 2 different sets of controls: one received a comparable inoculum of normal liver, the other was not injected. Following ether administration, the liver and sometimes the blood were collected at intervals of 3, 6, 9, 12, and 15 hours. The livers of the remaining infected animals were collected as the animals became moribund. The tissues

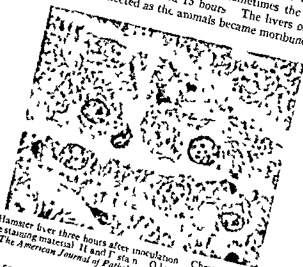


FIGURE 1. Hamster liver three hours after inoculation. Chromatin fragments are interspersed with pale staining material. H and E stain. Oil immersion. ×1134. Reproduced by permission from *The American Journal of Pathology*.

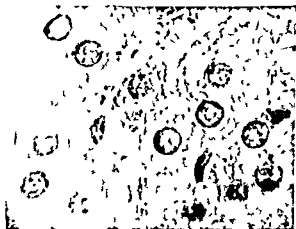
removed at the several intervals were biopsied and fixed in formalin and Zenker's fluid for H and E and Feulgen studies. The remainder was frozen and stored at −48° C for subsequent isolation of nuclei and study of the growth cycle.

Morphologic studies. Pertinent observations with H and E and Feulgen stains will be noted in FIGURES 1 to 5. At 3 hours the chromatin was distorted, sometimes fragmented and interspersed with pale pink staining material. The nuclear changes were more definite at 6 hours with peripheral margination of chromatin and single to multiple inclusions were scattered about. Considerable variation in the appearance of inclusion material was observed at 9 hours. In general, the changes were more advanced. Few to moderate numbers of cells resembled the 3- and 6-hour forms while the majority of nuclei were filled with basophilic material. Nucleoli usually were not recognized. In from 12 to 15 hours almost every nucleus was filled with basophilic material. Sections taken from animals sacrificed from 18 to 24 hours were very difficult to

distinguish from those sacrificed from 12 to 15 hours and often from those sacrificed from 9 hours In scattered areas nuclear changes were infrequently more variable The majority of cells contained full inclusions, others were



FIGURE 2 Hamster liver 6 hours after inoculation Inclusion material is more extensive and darker staining in some cells in others 3 hour changes predominate H and E stain Oil immersion ×1134 Reproduced by permission from *The American Journal of Pathology*[18]



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adherent along certain areas of the periphery and were retracted in some degree Scattered classic inclusions were present in some sections and absent in others The staining of inclusions varied from basophilic to eosinophilic Reissig and Melnick[18] have reported that, after appropriate fixation and immersion in alcohol, nuclear material occurring in infected cells retracted and appeared as inclusion bodies

The Feulgen nuclear reaction was not evident in 3 hours Single or multiple inclusions that evidenced pale to moderate staining Feulgen reactions were noted at 6 hours At 9 hours the relatively few Type A inclusions were usu-

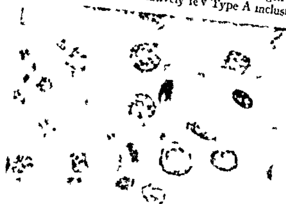


FIGURE 4 Hamster liver 6 hours after inoculation Two nuclei in lower right contain conspicuous Feulgen positive material the larger of which appears partially retracted Margination of chromatin is conspicuous many cells in the field are out of focus Feulgen stain Oil immersion ×1134 Reproduced by permission from *The American Journal of Pathology*[16]



FIGURE 5 Hamster liver 15 hours after inoculation Field is typical of 12 and 15 hour changes The inclusions are of dense appearance and markedly positive completely filling the nucleus in most instances Feulgen stain Oil immersion ×1134 Reproduced by permission from *The American Journal of Pathology*[16]

ally pale to medium staining rarely strongly positive The most abundant were full inclusions and ranged from pale to strongly positive the latter being more numerous At 12 and 15 hours the over all reaction was more uniform and the majority of nuclei were filled with markedly positive Feulgen staining material At 18 to 24 hours the size of the inclusions and the intensity of the reaction were less uniform in some areas of the slide under study However,

it deserves emphasis that often it was impossible to distinguish between the changes seen from 9 through 24 hours The observations are, at best, rather broad generalizations

Growth cycle of L 11 Dilutions of blood and liver obtained from animals at the previously stated intervals were titered in hamsters three weeks of age and the LD_{50} determined by the classic procedure of Reed and Muench[19] The results are illustrated in TABLE 1 The data relating to the liver are representative of a number of different trials The blood was difficult to obtain in sufficient quantity and only two trials were complete enough for comparison It is apparent that viremia was present through the period studied and would contaminate the liver However, enough differences emerge to permit a reasonable comparison Under the conditions of the experiment the virus obtained from the liver rose steadily in titer from 3 to 12 hours In either case the virus content reached a maximum at from 9 to 12 hours

It seems valid to state that the sequential development of nuclear changes from 3 to 12 hours is concurrent with the phase of increasing virus titer between

TABLE 1
TITER OF DETECTABLE VIRUS IN LIVER AND BLOOD

Time (hours)	Blood		Liver	
	P 124	P 135	P 124	P 135
1	4 0	Not done	Not done	Not done
3	3 5	5 2	3 7	4 0
6	Not done	4 5	5 3	5 5
9	5 0	6 0	6 8	7 0
12	5 7	7 0	8 0	8 0
15	Not done	7 0	8 0	8 2

The results of hamster titrations are expressed as the negative log of the LD_{50} P represents the passage number

3 and 12 hours, however, the most significant changes occur between 6 and 12 hours Also, at this time the majority of nuclei become filled with Feulgen positive material

These results are very similar to the data obtained by Scott *et al*[4] It is not meant to imply that, although the morphologic changes are closely related to the growth cycle, these nuclear changes represent developmental forms of the virus It is suggested that these changes reflect an expression of abnormal cell metabolism, a manifestation of cell injury

Changes in nucleic acid and protein in hamsters Much of this phase of the work has been reported elsewhere[17] Livers from the first-mentioned study

interference[20] Protein was determined by a modification[17] of the biuret method of Robinson and Hogden[24] The dry weight of nuclei is expressed in arbitrary units of 10^{-9} mg The average weight of nuclei from uninoculated control

animals was 52 and did not vary significantly from the inoculated controls (animals receiving normal liver). The infected nuclei increased in weight between 9 and 15 hours to an average figure of 90. For the same time period protein and RNA changed from approximate average values of 32 to 54 and 2.8 to 4.4 × 10^{-9} mg, respectively. DNA values for control and infected animals could not be distinguished statistically, and the average was 10.7 × 10^{-9} mg/nucleus. The various differences between infected and control nuclei are indicated in FIGURE 6 as percentages of change from normal controls. It has been shown that the most significant morphologic changes occur between 6 and 12 hours and are concurrent with the growth cycle. The chemical

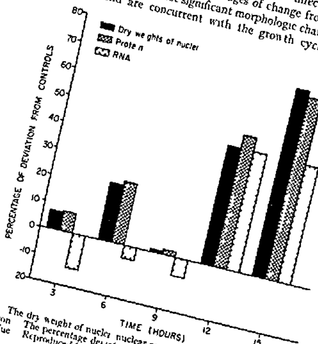


FIGURE 6. The dry weight of nuclei, nuclear protein, and RNA at various time intervals after inoculation. The percentage deviation from normal (control) is the change divided by the normal value. Reproduced by permission of *The American Journal of Pathology*.[11]

changes lagged somewhat, possibly due to the limitations of the methods. These effects reached a maximum between 9 and 15 hours. As yet nothing is known concerning the qualitative nature of either the protein or nucleic acid or whether they are associated. Recent data may indicate that they are combined in some way.[25] In our research, inclusions are stained only moderately with mercuric bromphenol blue as employed by Mazia *et al*.[26] However, following extraction of the nucleic acids with hot trichloroacetic acid, the inclusions stain deeply.[25]

Increase in total liver lipids in FAV infected hamsters. As part of the study to investigate the chemical pathology of hepatitis in this host it seemed pertinent to study lipids. A report of a preliminary study is mentioned.

Following IP inoculation of 20 to 25 male Syrian hamsters with 1 ml each,

of a 20 per cent suspension of infected livers, 3 animals were selected at random at 3, 6, 9, 12, and 15 hours and etherized at these times Three uninoculated animals for each passage served as a source of control tissue The livers were perfused with physiological saline via the portal vein, removed, forced through a 50-ml syringe into a beaker, weighed, and lyophilized The dried pooled tissues were reweighed and extracted at room temperature with 3 changes of 20 ml of chloroform methanol (2 1 by volume) during 24 hours The combined extracts were chilled and evaporated to dryness The dry residues were extracted at room temperature with 3 changes of 7 ml of petroleum ether for 30 to 45 min each The petroleum ether extracts were pooled, evaporated with a stream of dry CO_2, and dried under vacuum to constant weight

Statistical analyses (student's t test*) indicate (TABLE 2) that significant increases occurred between 0 and 9 hours ($p < 0.05$) and between 9 and 12 hours ($p < 0.02$)

TABLE 2
LIPID CONTENT OF HAMSTER LIVERS AS PERCENTAGE OF DRY WEIGHT

Time after inoculation (hours)	Animals inoculated with suspension of normal livers (percentages)	Animals inoculated with suspension of infected livers (EAV)* (percentages)	Change (percentages)	p†
0‡	16 4	15 4		
			3 5	<0 05
9	10 4	18 9		
			5 4	<0 02
12	14 0	24 3		
			0 3	>0 05
15	11 0	24 0		

* Equine abortion virus
† Student's t test
‡ Uninoculated control

Chemical Changes in HeLa Cells Infected with EAV

It is well understood that tissue culture appears to offer a model system for biochemical and other studies of animal viruses Fortunately, toward this end EAV was successfully adapted to the HeLa cell[7] which, concurrent with infection, develops conspicuous intranuclear inclusions not noticeably different from those seen in hamster liver Cytopathogenic effects are more or less complete in 48 hours, with sloughing of the cells from the glass Due to difficulties inherent in the problem that have not been overcome, the morphologic sequence of infection cannot be correlated with the growth cycle with any certainty However, interesting and provocative biochemical changes which have been reported elsewhere,[28, 29] occur in the infected cell RNA DNA and protein values were determined on whole cells, both infected and control values are expressed on a per cell basis In FIGURE 7 the percentage deviation of infected cells from controls is presented The data[29] subjected to statistical analysis by student's t test indicate that RNA was not changed, whereas DNA ($p < 0.01$) and protein ($p < 0.01$) increased significantly Of course, no corre

* Student's t test for significance If $p \leq 0.05$ the change is considered significant

lation with the growth cycle is implied It is possible that, by use of cell fractionation techniques, nuclear and cytoplasmic RNA and protein may show significant changes Such experiments are in progress

The results of the studies with hamster hepatitis indicate interference in the biochemistry of the cell, and several of the changes during the course of infection have been measured It is reasonable to assume that a nucleus that contains such a conspicuous mass of material that can readily be stained by a variety of methods should add some mass to the cell

It is interesting to note that with the methods employed not all of the nuclear mass has been accounted for *This unidentified material increased significantly* with infection [17] It may be inferred that the composition of the inclusion consists, at least in part, of protein and DNA, as suggested by the results of staining with mercuric bromphenol blue and the Feulgen reaction The fact that the inclusion stains conspicuously by the Feulgen technique, while the total amount of DNA per cell is not increased, may be a contradiction of the usual

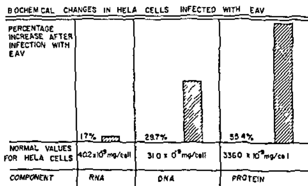


FIGURE 7 Biochemical changes in HeLa cells infected with EAV

interpretation of the Feulgen reaction A possibility, in the realm of speculation, is worth considering to explain the uniform Feulgen reaction of the in

munication), who was not able to demonstrate any increase in DNA by the

value found by biochemical analysis [27]

The relationship of the virus to the inclusion in this instance was purely speculative until Bracken and Norris[30] reported that a study of electron micrographs of EAV infected hamster liver revealed small aggregates of intranuclear virus particles that were not equivalent to the intranuclear inclusion visualized by light microscopy

Tajima and Motohashi[31] have demonstrated that intranuclear inclusion bodies occurring in canine hepatitis were composed of amorphous matrix mixed with numerous virus particles. The claim is made that the matrix appears to have been derived from chromatin and has been utilized for the formation of the observed virus particles.

The results of Feulgen study certainly do not permit any claim as to the composition of the virus. If the virus particles from the hamster contain DNA the total amount of which is in excess of the normal amount per nucleus it is not probable that the amount of virus DNA elaborated could be detected due to the limitation of the method of analysis.

Bloch and Godman[32] have reported a cytological and cytochemical (Feulgen microspectrophotometric) study of human viral papilloma. In the earliest stage eosinophilic nucleic acid free intranuclear inclusions are identified later becoming basophilic and Feulgen positive. These authors claim that in a later stage in the evolution of the cellular lesions all the DNA of the cell is

Sections from the same cell have been compared by the Feulgen method and electron microscopy and it has been stated that since the observed Feulgen positive crystals are composed of virus particles it is likely that the virus contains DNA[33, 34]. In Type 5 adenovirus infection intranuclear crystals are Feulgen negative whereas surrounding virus particles are positive[34, 35].

In the HeLa cell DNA is significantly increased whatever the mechanism may be. Presuming that the virus replicated in the cell in question contains DNA it is unlikely that the total increase could be accounted for on this basis as an improbable number of virus particles would be required to equal the increased DNA per cell. It deserves some emphasis that adenovirus[10] FAV[23] and herpes virus[12] infections of HeLa cells are associated with conspicuous inclusions and that in each case the DNA content is increased. On the other hand infection of HeLa cells with poliovirus results in increase of cytoplasmic RNA[13] however Hydén found an increase in nuclear RNA and a decrease in cytoplasmic RNA. If true these data suggest that perhaps the type of cell as well as the virus may influence the nature of the cellular reaction.

The apparent constancy of DNA in nuclei derived from infected hamster liver as contrasted with the increase in HeLa cells is unexplained. It is speculative to suggest that the increase in DNA may be related to the malignant cell. In cases where DNA is increased changes in ploidy should be considered this has not been excluded in the HeLa cell infection.

It is of interest to note the results obtained with several chemical compounds. Laird[16] isolated rat liver nuclei from animals previously injected with thioacetamide. In this study the DNA remained constant while RNA increased 3 5 times and protein 2 5 times. Griffin *et al*[27] reported that the DNA and protein content of rat hepatic cells increased during azo dye carcinogenesis.

The nature of protein increase occurring in hamster nuclei and HeLa cells is unexplained and in either case is of such magnitude as to suggest protein synthesis. Inclusion bodies resulting from another virus infection have been shown to contain protein but in what amounts has not been stated. Bloch

and Godman[38] have demonstrated that nuclear inclusion bodies contain a basic protein of the histone type In several instances of infection, crystalline protein occurs in the nucleus and is free of DNA [33, 34]

Changes in Tissues Infected with Fowlpox Virus

The literature of fowlpox has been reviewed by Todd[39] Goodpasture[40] a pioneer in the study of this disease, has demonstrated that lipids accumulate and can readily be stained in lesions of the chick scalp and chorioallantoic membrane (CAM)

The morphology of the lesion has been studied with H and E stain[39] and by the Feulgen technique[41] The inclusions were Feulgen negative until 72 hours, thereafter they remained positive as long as the study continued (12 more days) The individual elementary bodies are conspicuously positive after lipid extraction

Mature lesions satisfactory for the purpose of this study were arbitrarily selected at 7 days and 5 days for the chick scalp and chorioallantoic membrane, respectively Judging from the common fat stains, the amount of lipid that accumulates in the inclusion is impressive The infected tissue seemed to be an excellent source for the study of lipids which might be expected to vary as a result of virus infection

Lipid studies have been undertaken with other virus infected tissues Cohn[42] reported decreased phospholipid phosphorus in influenza infected CAM Cornatzer *et al*[43] examined papillomata from rabbits infected with Shope virus and reported a diminution in total lipid and an increase in phospholipid phosphorus

Recently Todd *et al*[44] have reported quantitative lipid changes in fowlpox infected tissues as indicated below Infected scalp and chorioallantoic membrane and suitable control tissue were lyophilized to constant weight and after preliminary extraction with alcohol and Bloor's solvent were continuously extracted in a Soxhlet apparatus with diethyl ether Combined extracts were evaporated to dryness under nitrogen and the residue extracted exhaustively with petroleum ether The various chemical determinations were performed by standard methods Summation of individual lipid fractions indicates that 105 per cent of the scalp and 101 per cent of CAM lipid were recovered this is within the experimental error of the method

The various lipid fractions from normal and infected chick skin are indicated in FIGURE 8 The total lipid contents were 23 95 ± 1 24 and 19 46 ± 0 65 per cent respectively and are calculated on the basis of tissue dry weight The various percentages of the constituents as shown are based on the total lipid value Statistical analyses indicate that total lipid and esterified cholesterol free and total fatty acids had changed significantly with p values $<0\ 05$ The decrease in total lipid may be more apparent than real as the dermis shows connective tissue proliferation and heavy infiltration by inflammatory cell thus probably increasing disproportionately the weight of the scalp tiss terms of percentage of extracted lipid

The data concerning CAM lipids are shown in FIGURE 9 The total of normal CAM weight were 15 98 ± 0 74 per cent and infected 23 7 per cent The other lipid fractions are expressed as percentages

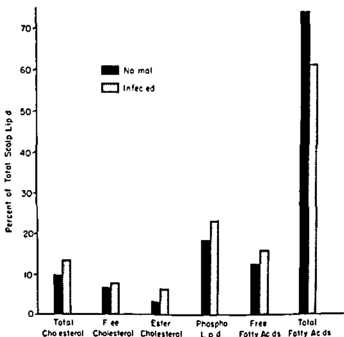


FIGURE 8 Lipid fractions from normal and infected chick scalp

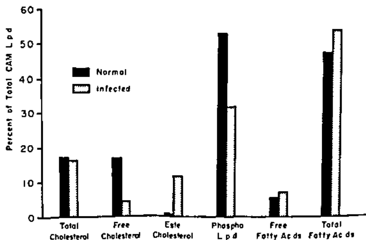


FIGURE 9 Lipid fractions from normal and infected chorioallantoic membranes

lipid The p values of total lipid free and esterified cholesterol and phospholipid were <0 001

The data seem to indicate that the lipid metabolism of tissue infected with fowlpox is altered and tend to incriminate the tissues (epithelial cells) bearing the most occult lesion the inclusion body The contribution, if any, of the contiguous dermis has not been determined as yet Other sites of possible metabolic alteration such as the liver should be investigated as these may be affected despite the fact that epidermal structures are the only tissues that show specific lesions since Goodpasture[40] has shown that viremia is present in the fowl

In conclusion information presented here concerning equine abortion virus and fowlpox and the work of other investigators make it evident that unexplained events occur in the virus infected cell These changes indicate significant alteration in the biochemistry of the cell Some of the components determined in the present study have diminished others have increased and in some instances probably greatly exceed values that might be accounted for solely by replication of virus particles Whatever the mechanism of these changes may be and how they are related to virus replication remains to be shown

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SOME BIOCHEMICAL EFFECTS OF INFECTION WITH FRIEND'S LEUKEMIA VIRUS

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The development of the cellular changes that a pathologist uses to character ize a disease is a step that is subsequent to and dependent upon prior altera tions in the biophysical or biochemical properties of the cells Certainly, this is no less true of virus infections than of other diseases Indeed in some virus infections there can be virus replication without apparent microscopic pathol ogy

In recent years our laboratories as well as those of others have reported some of the early biochemical alterations seen in tissue culture prior to the onset of pathology [1 2 3]

With regard to *in vivo* infection, the agent described by Friend[4] as producing leukemia in mice has several attributes that commend it for a study of early effects of a virus agent In the early stages it produces a disease restricted to the spleen so that secondary physiological alterations probably are mini mized It produces disease rapidly pathological alterations can be seen in four or five days The disease picture produced is quite reproducible in a very high percentage of injected mice, and the mice survive for a fairly long period of time, so that experimentation and observation can be continued Incidentally we refer to this disease as leukemia although we realize that ome competent authorities do not agree with this designation

We were interested in trying to detect some metabolic change in mouse leen cells prior to the onset of detectable pathology It was felt that such ry early changes would be useful in gaining insight into the biochemical nature of the disease process There are, of course innumerable individual metabolic processes that one might choose to examine To attempt to cover one by one any significant part of these processes would be a formidable task Instead we chose to survey in one operation a broad area of metabolic activ ity namely phosphorus metabolism as manifested by radioactive phosphorus uptake It was felt that if infection produced a change in the uptake of P32 by the spleens one could attack the problem in greater detail to determine if a more intimate picture of the altered biochemistry could be found

The first part of this paper deals with our observations with whole tissue and the second part with some of the more detailed biochemistry

In order to determine if there was any alteration in phosphorus uptake as a result of infection with the Friend virus a number of experiments were per formed essentially as follows approximately 150 white female NIH general purpose mice approximately 18 gm in weight, were inoculated intravenously with 0 2 ml of a cell free 10 per cent extract of spleens from mice carrying the disease The titer of the extract was about 10^{4} ID_{50} 0 2 ml An equal number of control mice were given a similar extract from normal mouse spleen Be ginning 1 day after inoculation and at intervals thereafter 6 infected and 6

control mice were chosen at random from the group and given 5 μc of P^{32} intraperitoneally. Three hours later these mice were sacrificed and their spleens removed, weighed wet, dried in preweighed stainless steel planchets reweighed, and their radioactivity determined. At the same time that the spleens were removed, pieces of liver were also removed. Blood from each group of 6 mice was also taken and pooled. Some of the data from 1 experiment of this type are summarized in FIGURE 1, where the abscissa represents the number of days postinfection, and the ordinate represents the average wet weight of the 6 spleens, as well as the averaged cpm/mg dry weight. It will be seen that the spleens started increasing in size after about 4 days, but the P^{32} uptake became higher in the infected animals after only 2 days. It may

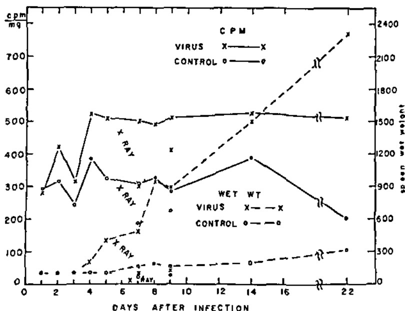


FIGURE 1. Effect of the Friend agent on uptake of P^{32} by spleen with and without X ray treatment.

be repeated here that this is not cumulative uptake of P^{32}, but rather represents just the P^{32} uptake in the 3 hours prior to sacrifice. FIGURE 2 shows another experiment where the same result can be seen. It might be remembered that at such a short interval after infection as 2 or 3 days, there can be seen little or no pathological alteration and that little virus can be recovered from the spleens.

There are a few other observations that should be mentioned. In the first place, one might ask whether the increased P^{32} uptake in the infected spleens is due to a possible alteration in spleen capsule permeability. The P^{32} is given intraperitoneally and if the spleen capsule permeability were increased as a result of infection one might anticipate the results obtained. However, when the P^{32} was given intravenously the infected tissues still took up more P^{32} per mg of tissue than did the uninfected ones.

Another point along the same line relates to a possibly higher specific radioactivity in the blood plasma of the infected animals. If such were the case, then a higher specific radioactivity in the spleens would be anticipated. Indeed, it would be anticipated for all the tissues of the infected animals. Liver and, in a few instances, brain from infected animals were examined and found to have no higher specific activity than had the controls. Direct measurement of the inorganic phosphate and the radioactivity of blood plasma and of the combined red and white blood cells revealed no consistent differences between the infected and control animals. This is illustrated in FIGURE 3. However, some evidence of a peculiar phosphorus metabolism is discernible in the wide

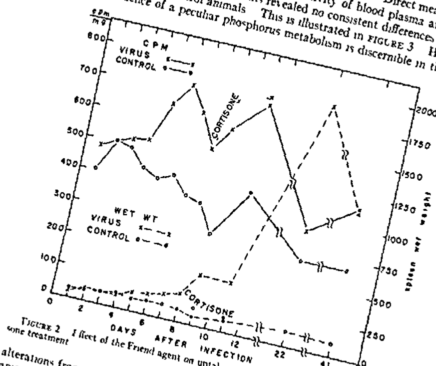


FIGURE 2. Effect of the Friend agent on uptake of P^{32} by spleen with and without cortisone treatment.

alterations from day to day in the radioactivity of the plasma of the infected animals. The controls were much more stable.

Accordingly, it was concluded that the most likely explanation of the stimulated P^{32} uptake seen in the infected tissues was an effect of the virus on the tissue. Was this stimulation only a reflection of an increased growth rate? Several lines of evidence to be brought out in this paper indicate that increased growth rate certainly was not the only factor involved although it may, indeed, be a contributory one. As one observation relating to the question of growth and P^{32} uptake, it may be seen from FIGURES 1 and 2 that the increase in P^{32} uptake preceded by 1 to 2 days the increase in spleen size. Possibly more convincing evidence can be obtained from the work with cortisone and X ray. As adjuncts to the experiment just described, these 2 agents and, later, chloram

bucil were administered in the hope of obtaining at least temporary remissions as with some human leukemias and lymphomas. Cortisone at a level of 1 mg/day was given on the eighth day of infection to some of the mice used in the experiment summarized in FIGURE 2. On the following day and on the day thereafter, 6 treated control and infected mice were tested for spleen P^{32} uptake at the same time as the untreated controls and infected mice. The results of this experiment are shown in FIGURE 2.

The infected spleens stopped growing and returned to normal size. In spite

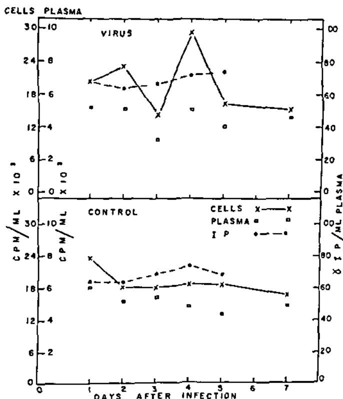


FIGURE 3. Effect of infection with the Friend agent on P^{32} content of blood cells and blood plasma and on plasma inorganic phosphate.

of this these infected treated spleens took up as much P^{32} per mg tissue as did the infected spleens from animals that received no cortisone. These results were confirmed in other experiments.

In the experiment represented in FIGURE 1, some of the mice were given 300 r of total body X radiation on the fifth day of infection. Here as in FIGURE 2 X ray caused the infected spleens rapidly to return to normal size instead of continuing to increase. The rate of P^{32} uptake decreased after X ray in both the infected and noninfected animals but the X rayed infected animals still took up more P^{32} than did the X rayed controls. As a matter of fact as seen in FIGURE 4 the percentage increase in P^{32} uptake was just as great after either

X ray or cortisone as it was with no therapy, even though the spleens had returned to normal size. One further observation along these lines should be mentioned. When cortisone was given beginning the day after virus inoculation, there was no sustained growth period in the infected spleens, but still the virus effect on P^{32} uptake was manifest, as seen in FIGURE 5.

What can be said about the reason for the continued stimulation of P^{32} uptake in these treated spleens? If, indeed, the spleens had been reduced to normal size, why did they not behave like normal spleens? In the majority of cases the X rayed spleens, although small, showed almost as large a percentage of abnormal cells as did the unirradiated ones, so that high P^{32} uptake

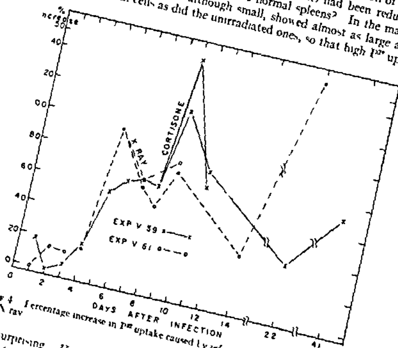


FIGURE 4. Percentage increase in P^{32} uptake caused by infection with and without cortisone and X ray.

was not surprising. However, in a few well-controlled observations, particularly after two doses of X rays, the remission was almost complete in that very few abnormal cells were present but a high P^{32} uptake was still found.* Further work will be needed to elucidate this finding, particularly along more detailed biochemical lines. The important observation from our viewpoint, is that spleen enlargement is not necessary for increased P^{32} uptake.

We did other experiments to test the specificity of the effect of this virus on spleen P^{32} uptake. There are quite a few reports in the literature on work done with transplanted leukemias,* but the experimental conditions used were somewhat different from ours. We decided to determine whether other diseases involving either spleen enlargement or spleen pathology would show a

* One to two weeks after X ray induced remission, the infected spleens again began to grow rapidly.

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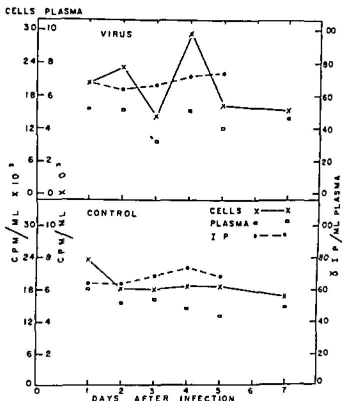


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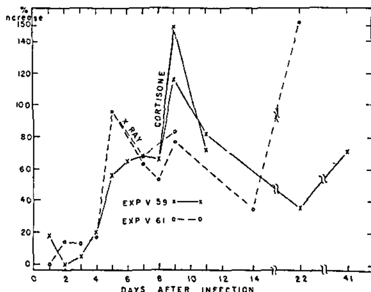


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* One to two weeks after X ray induced remission the infected spleens again began to grow rapidly

similar picture to that observed with the Friend virus. The diseases studied are shown in TABLE 1 which shows that under our conditions the only additional diseases that showed increased uptake of P^{32} into the spleen were the spontaneous AKR leukemia and the lymphocytic leukemia produced by Gross's virus. In other words an increase in spleen size, or pathological alterations, or even transplanted leukemia was not a sufficient condition to cause the increased P^{32} uptake that we saw with the Friend virus.

FIGURE 6 represents a photomicrograph taken of infected spleens stained by fluorescent antibody to the viral agent, by the direct procedure. Fluorescein labeled globulin from immunized rabbits was used to stain impression smears of spleens from infected mice. All sera were absorbed twice with normal liver

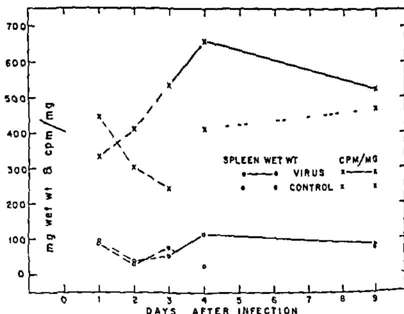


FIGURE 5. Effect of early treatment with cortisone on increased P^{32} uptake by spleens of animals infected with the Friend agent.

powder and twice with normal spleen powder, and were treated with Dowex 2 × 4. FIGURE 6 illustrates the cytoplasmic staining seen in infected spleens. In some of the cells a nucleus and a cell membrane can be seen fluorescing. In the actual slides the nucleus was blue and the cytoplasm was yellow. Infected spleens stained with labeled globulin from rabbits immunized with normal mouse spleen and normal spleens stained with labeled antinormal spleen globulin or antiviral globulin showed no significant cytoplasmic staining and only occasional nuclear staining.

During the remainder of this paper we shall describe briefly some of the biochemical studies we have done so far with these spleens. We first studied the distribution of the injected P^{32} into several biochemical fractions. The procedure was essentially as follows: 10 to 20 mice were infected as usual by intravenous inoculation of a cell free filtrate from infected spleens. A similar num

TABLE 1

EFFECT OF VARIOUS PATHOLOGICAL CONDITIONS ON UPTAKE OF P^{32} BY SPLEENS AND THYMUS

Condition	Average wet spleen weight (mg)		CPM/mg dry weight		Percentage change
	Infected	Control	Infected	Control	
Lymphocytic choriomeningitis	116	127	248	218	14
Histoplasma capsulatum	503	247	285	248	13
Lymphocytic leukemia (transplanted) spleen	263	68	640	945	−35
Leukemia P 195 (Hodgkin's-like transplanted)	613	187	62	62	0
Leukemia HP 11764 G 4 (transplanted)	154	147	49	44	11
Spontaneous AKr leukemia					
spleen	415	75	123	67	83
thymus			174	67	160
Gross virus-induced leukemia					
spleen	751	57	543	322	69
thymus			396	248	60

At least 6 experimental and 6 control mice were used and in most cases 2 or 3 such groups were used. Differences of less than 20 per cent are not considered significant.

FIGURE 6. Photomicrograph of infected spleen smear stained by fluorescent antibody technique.

ber of other animals received a normal spleen filtrate. After the desired period of infection the animals were given P^{32}, 3 hours later they were sacrificed and their spleens fractionated by established methods. The fractions studied and the determinations performed are indicated in FIGURE 7. From these data we get the concentration of each type of phosphorus in the tissue, the specific radioactivity of each fraction, and the concentration and specific radioactivity of the RNA and DNA. This type of observation was carried out a number of times and at different stages of infection.

For simplicity, we have summarized, from the rather large amount of data, what we feel are the most important features. TABLE 2 shows the percentages

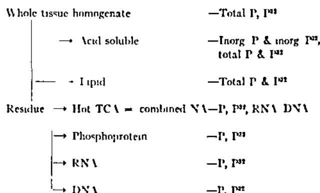


FIGURE 7. Diagram of fractionation procedure and analyses performed on each fraction.

TABLE 2
PERCENTAGE INCREASE* IN VALUES FOR INFECTED SPLEENS OVER THOSE OF CONTROL SPLEENS

	P concentration		Specific radioactivity	
	4 days	10 days	4 days	10 days
RNA	61	171	93	69
DNA	0	104	12	283

* Not absolute values.

of change induced by infection in the ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) concentrations and in their specific radioactivities for one case in which there had been relatively little ... extensive

was

a 61 per cent increase in RNAP concentration and a 93 per cent increase in its specific activity as compared with the values from noninfected spleens. Similarly at 10 days, when there had been considerable spleen enlargement, the infected RNAP and its specific activity were also higher than the control values. However, alterations in DNA do not appear until the spleens really had begun to enlarge. This is consistent with other literature reports in which DNA

metabolism is found to be slight in non growing tissues but becomes marked when cell division takes place.[6,7]

We should add that the specific activity of the lipid P fraction was also elevated in the early stages of the infection.

To revert to the question raised earlier about the relationship of increased P^{32} uptake to growth, these data suggest that the effect of growth is to increase the uptake of P^{32} into the DNA, whereas increase in size is not necessary for an increase in RNA concentration or specific activity. Of course, there could also be a virus effect on DNAP, but at the moment we should not be able to detect it.

Other evidence confirming and amplifying these observations came from experiments in which radioactive infected and control spleens were separated into nuclei, mitochondria, microsomes, and so-called soluble fraction (or subparticulates) which, in turn, were fractionated into their biochemical components, namely the acid soluble, lipid, nucleic acid, and phosphoprotein fractions. In order to simplify very complicated data for presentation here, we have prepared TABLE 3 in such a way as to show only the percentage increase

TABLE 3

PERCENTAGE INCREASE IN SPECIFIC ACTIVITY OF BIOCHEMICAL FRACTIONS OF SUBCELLULAR COMPONENTS: THREE-DAY INFECTION WITH NO SPLEEN ENLARGEMENT

Fraction	Lipid	Nucleic acid
Nuclei	0	0
Mitochondria	0	0
Microsomes	20	30
Subparticulates	80	40

caused by infection in the specific radioactivity of the more interesting components. In other words, we proceeded as follows. The biochemical components of the subcellular particles from infected and control spleens were separated. The amount of radioactivity and the amount of P in them was determined, and the specific activity of these fractions as cpm/mg P was calculated. The percentage differences between infected and control components were calculated; these calculations are presented in TABLE 3.

These data are obtained from an infection of three-days duration. There was no increase in spleen size at this time. Examination of the summaries in the table shows that the nuclei and the mitochondria from infected tissue showed no changes in either the lipid or nucleic acid fractions as compared with controls. The microsomes and subparticulate elements from the infected spleens, on the other hand, showed significant increases in the specific activity of the lipid and nucleic acid fraction as compared with the control values. In the latter two fractions, the microsomes and subparticulates, the nucleic acid is RNA. These data show again that it is the cytoplasmic RNA that is stimulated early in infection, while the DNA is not.

Current evidence suggests that the microsome and subparticulate fractions perform protein synthesis[8] and, to some extent, RNA synthesis. The combination of microsomes and subparticulates in a suitable *in vitro* system can

out protein synthesis,[9] while a *p*H-5 2 precipitate from the subparticulates can add end groups to suitably prepared subparticulate RNA[10] It would appear from our data that, at least with respect to phosphorus, the earliest effect of the virus is to stimulate these protein- and nucleic-acid-synthesizing components Other data not presented show that, almost simultaneously, there is an increase in activity in the acid soluble parts of the mitochondria, the energy supplying system

A little speculation, based on these data, may prove useful One may consider the situation in a normal cell to be such that a sort of mass action law type of equilibrium exists between nucleic acid and nucleic acid precursors, as well as between the protein for which this nucleic acid is a template and the protein precursors When the virus infects the cell, there is good reason to think that one of the first things it does is to divest itself of its protein, if it had not already done so before entering the cell The viral nucleic acid then may or may not break down into smaller components We shall consider the smaller component concept first If we were to hypothesize that these smaller components attach themselves to the subparticulate RNA of the cell, possibly through the mediation of the *p*H-5 enzyme just mentioned, then the equilibrium situation is destroyed There are new templates for RNA and thence for protein, and new demands are set up but these are now demands for virus RNA and virus protein The cell knows only that it needs to make more RNA

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Acknowledgments

We thank Ruth Kirschstein for the pathological evaluations provided and Victor Haas, Hubert Hasenclever, Lloyd Law, and Ludwig Gross[11] for supplying many of the animals used

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CYTOPATHOGENIC EFFECT OF TWO NEWLY RECOGNIZED MYXOVIRUS STRAINS MECHANISM OF SYNCYTIAL FORMATION

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The recent isolation of numerous newly recognized myxoviruses[1 2] demonstrates the heterogeneous composition of this group, since they were found in patients ill with diseases such as mumps, influenza, or croup Myxoviruses may produce in different tissue culture systems (monkey kidney cells, Kb cells) different manifestations that may be described schematically as follows (1) little or no cytopathogenic effect but the proliferation of the virus may be detected by the hemadsorption technique, as described by Vogel and Shelokov[4] and (2) definite cytopathogenic effect (CPE) At present two different types of CPE may be recognized the development of virus induced giant cells and the development of intracytoplasmic inclusion bodies These two types of lesions may be found either associated or alone, this property depends on the virus strain examined and also on the tissue culture system chosen for the propagation of the virus

Recent studies in this laboratory were focused on the mechanism of cytological alteration produced by myxoviruses Two recently isolated myxoviruses were chosen for their capacity to induce typical or rapidly extending cytological lesions the Greer strain of CA virus isolated and described by Chanock, and the Mills strain of the HA 1 viruses isolated by Rowe and Huebner and recently identified by Chanock [2] These were used as models for this study For comparison a similar virus, apparently belonging to the HA 1 virus group and isolated in March 1958 in this laboratory, was also used[3] The study was carried out with microcinematographical and cytochemical methods

MATERIAL AND METHODS

Monkey (cynocephalus) kidney cells were grown on coverslip preparations in Leighton tubes with a medium containing 45 per cent Hanks' solution 50 per cent modified 199 medium, and 5 per cent inactivated horse serum The same medium was used for maintenance, but without serum

Kb cells were grown in casein hydrolyzate medium[5] with 10 per cent horse serum in the maintenance medium horse serum was replaced by 2 per cent chicken serum

Mills strain of the HA 1 virus was propagated in Kb cells ($^2/_{10}$ ml containing 100 CPD_{50}) and checked under the microscope every 6 hours to detect early cytopathogenic effect When early lesions appeared, the coverslip was mounted in a plastic chamber and sealed with paraffin Progression of the lesions was observed at 37° C with a Zeiss microscope and pictures were taken

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at a speed of 6 frames/min Uninoculated control Kb cells were observed under the same conditions The observation period averaged 18 hours

Cytochemical Techniques

Nucleic Acids

Feulgen staining After fixation with Helly fixative and elimination of Hg with Lugol's iodine solution, the slides were hydrolyzed for 8 min with HCL (N) at 60° C, and then washed in distilled water at 4° C to stop the reaction The slides were immersed in Schiff's reagent for 90 min and rinsed 3 times in a solution of sodium bisulfite (10 per cent), 10 ml HCL (N), 10 ml, and distilled water 200 ml

*Methyl green-pyronine and ribonuclease test** Fixation in acetone for 2 hours at 4° C The coverslip was mounted on 2 matchsticks on a wet filter paper, and the whole was placed in a Petri dish The preparations were immersed in a ribonuclease solution 1 5000 in phosphate buffer at *p*H 7 at 55° C for 1 hour Control coverslip preparations were placed in the same conditions and covered with a phosphate buffer solution Both coverslips were then stained with a methyl green pyronine stain for 30 min and washed quickly with ethanol acetone, 50 50, followed by pure ethanol

Toluidine blue and ribonuclease test The same procedure was used, except that the coverslips were stained with toluidine blue 0 1 per cent in an acetic buffer (M/15) at *p*H 3 and at *p*H 4

Proteins

Ninhydrin reaction (Romieu procedure) Preparations (Lison) were fixed in 10 per cent formalin fixative and placed in boiling 0 4 per cent ninhydrin solution for 1 min Cells were observed after mounting in glycerin

*SH groups** Coverslips were fixed in 10 per cent formalin containing 5 per cent trichloracetic acid (TCA) for 1 hour or more and stained without any fixation Two tests were performed simultaneously in cells with blocked SH functions blockage being produced by using $HgCl_2$ for 1 hour and in cells with free SH functions, which were subjected to 3 baths 5 min each of K ferricyanide (0 1 per cent), 25 ml and $Fe_2(SO_4)_3$ (1 per cent) 75 ml in the dark After washing in distilled water preparations were mounted in Canada balsam

Lipids

After fixation with 10 per cent formalin cells were washed with 70 per cent ethanol and stained for 15 min with a saturated solution of Sudan black B in 70 per cent ethanol

Polysaccharides

Preparations were stained by the *p* aminosalicylic acid (PAS) method after fixation in Helly solution or 90 per cent ethanol and 10 per cent formalin Cells were oxidized for 5 min with 5 per cent periodic acid as described by McManus* The presence of glycogen was eliminated by the amylase test

Incorporation of 35S Methionine

For the study of incorporation of 35S methionine 0.25 μc of *dl* 35S methionine/2 ml of tissue culture medium was added. After 2 hours of incubation the cells were fixed with 10 per cent formalin. Autoradiography was performed using an Ilford G5 emulsion. After 24 hours exposure in the dark at 4° C the plate was developed with the Kodak D 19b stock solution and fixed. Staining of the nuclei was performed with Mayer's hematoxylin.

Results

The production of multinucleated cells. Altogether six series of experiments were performed. In all of the six tissue culture passages in this laboratory the Mills strain of the HA 1 virus group induced the formation of giant cell. No intracytoplasmic or intranuclear inclusions were found in H and E stained preparations. The Feulgen reaction did not demonstrate any abnormal concentration of desoxyribonucleic acid (DNA) in the nucleus at the early stages of the syncytial formation. In the late stages some of the nuclei in the clumped syncytial areas showed the presence of some pyknotic nuclei. Neither did the methyl green pyronine stain show after the ribonuclease test any abnormal concentration of ribonucleic acid (RNA) when compared with control preparations.

In unstained preparations the development of giant cells was observed by microscopic and cinematographic techniques. One hundred DCP_{50} of virus was used in these experiments. The giant cells appeared in any area of the cell sheet (FIGURES 1 and 1*a*). During the early stages the number of the nuclei contained in each of the syncytial areas was limited to a few. At the time of extension of the giant cells more and more nuclei were gradually enclosed in the syncytium (FIGURES 2 and 2*a*) and in more advanced stages several hundred could be numbered. Thus in 48 hours about 50 per cent of the cell area showed typical alterations. After 48 hours some of the syncytia started to

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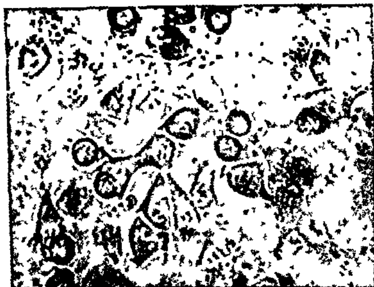
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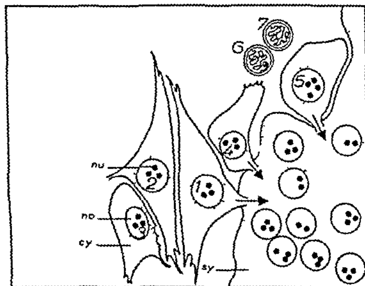
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is to be noted that no mitotic figures were observed on the syncytial areas although they were frequently seen in the control tubes and in the noninvaded region of the test tubes. Similar lesions were observed with the FA 102 strain isolated in this laboratory, a strain of virus that probably also belongs to the HA 1 group.

The number of the syncytial areas was proportional to the titer of the virus present in the culture under experiment and was remarkably constant from one experiment to the other. It has been postulated therefore that each of the syncytial areas is produced by one infectious unit.

As shown by the time lapse motion pictures the mechanism of giant cell formation is not atypical mitosis as sometimes suggested but a cell agglutination or conglomeration that may be described as cytophagia. A similar phenomenon has already been observed and described with some adenovirus strains, especially those with slow growth.[10]

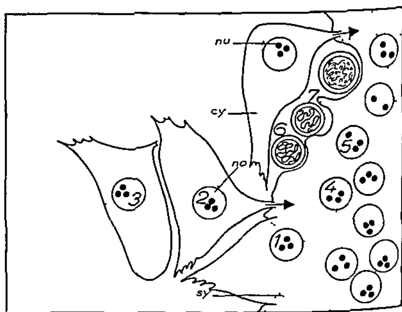




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FIGURES 2 and 2*a*. Frame from microfilm showing Kb cells infected with Mills strain of HA 1 viruses; cells 1–4 and 5 are already included in the syncytium; mitotic cells 6 and 7 are resistant to the cytophagia; after falling of the syncytium these will remain attached to the glass.

There is an increase in cytoplasmic volume at one extremity of the virus-parasitized cell. Contact with a neighboring cell produces attraction of the latter. When the two cytoplasmic membranes are in contact, a narrow communication is established between the two cells. The cytoplasmic content of the invaded cell is attracted into the apparently virus parasitized cell. Shortly afterward the nucleus of the cell is incorporated without any obvious morphologic damage to it. During the progress of the giant cell formation, some of the cells resist cytophagia. In some instances these cells show mitotic figures. In late stages, when the syncytium detaches itself from the glass these resistant cells still remain attached to it.

The cells included in the syncytia do not show any sign of cellular death. When stained with 0.04 per cent erythrosin no penetration of this stain in the syncytial areas was observed after 1 hour. After 48 hours, the *pH* of the test tubes seemed to be slightly more acid than that of the control tubes. As shown by the autoradiographic experiment, the 35S methionine apparently was incorporated in greater amount in the syncytial areas than in the noninvaded areas and the control cells.

Cytochemical Study of Cytoplasmic Inclusion Bodies

Nine series of experiments were performed and thus over 160 stained preparations were examined.

CPL produced by the croup associated (CA) virus has been described by Chanock in unstained preparations:[1] "In monkey kidney monolayer tissue culture this virus produces syncytial areas with loss of the cell boundaries followed by the breaking away from the surrounding cell sheet." Then small vacuoles make their appearance and the cell loses its structure, producing a picture best described as spongelike.

In hematoxylin and eosin stained preparations from the first passage, we observed syncytia containing eosinophilic intracytoplasmic inclusion bodies that were not present in the uninoculated tissue cultures. These lesions were regularly present in the nine subsequent passages performed in this laboratory (Figures 3, 4, and 5).

In the early stages of the progression of the lesions inclusion bodies were of a small size rounded and clearly limited. *In* more advanced stages, several inclusions were observed in the same cell and, in some instances the fusion of these inclusions resulted in a larger inclusion always sharply limited surrounded by an unstained area of irregular shape and frequently situated close to the nucleus. When high magnification with immersion oil is used, these eosinophilic inclusions showed a granular structure. No change was observed in the staining affinity of the inclusions in late stages when H & E staining is used. Eosinophilic inclusions were also *observed* in the cytoplasm of noninoculated control cells but they were not transmissible, they were much smaller and far less numerous than the inclusions observed in the infected cultures.

While this study was in progress Brandt[10] reported similar observations with Newcastle virus and with some strains of mumps virus. Both of these viruses belong to the myxovirus group. The Swiss cheeselike appearance described

the small eosinophilic inclusions sometimes present in the control tubes were not clearly demonstrated in this series.

The ninhydrin reaction was weakly positive, but a striking fact was the strong positivity of the SH groups as demonstrated by the Chevremont-Frederic reaction. The significance of this finding will be discussed later.

The PAS test showed the presence of polysaccharides or lipids in the inclusions in a very irregular manner. The PAS reaction was unaffected by amylase digestion and by toluene extraction. Sudan black B staining showed only a very small amount of lipids in the inclusions.

Discussion

Insofar as we know, the first observation of viral cytophagia in tissue culture was described in the P 134-4 strain of adenoviruses in HeLa cells by the simple study of stained preparations.[11] In the case of adenoviruses (chiefly slowly growing strains), agglutination by a virus parasitized cell of another healthy or [illegible] of the invader, [illegible]

viruses, and how a virus can induce in a cell such a powerful cytophagic activity, since many of the syncytia finally contain several hundreds of nuclei. Another striking fact is the absence of mitosis in the syncytial areas associated with an apparently high metabolic activity of the invaded cells, at least in early stages of the cellular injury.

tissue culture by physical and chemical agents, such as Koch bacillus extracts,[12] but in such cases none of them is transmissible by filtrates. Intracytoplasmic inclusions observed in our series are very similar to those described with measles. More information is still needed to evaluate the complete significance of these findings, but it seems worthwhile to consider the type of cell injury as a definite part in the characterization and description of any virus.

Summary

Recent reports on the isolation of newly recognized myxoviruses have shown the heterogeneous composition of this group. Myxoviruses may produce such unrelated clinical manifestations as mumps and respiratory infections. In different tissue culture systems (cynocephalus monkey kidney or Kb cells) myxoviruses may show either (1) proliferation without cytopathogenic effect but hemadsorption, (2) multinucleated giant cells, or (3) intracytoplasmic inclusions with or without giant cells.

A study is made on the basis of cinematographic and cytochemical observations of the mechanism of CPE produced in the latter two cases.

Two newly recognized myxoviruses, HA 1 and CA, have been studied as models. The HA 1 virus induces the production of multinucleated giant cells but no inclusion bodies; this giant cell formation is the result of cytophagia as demonstrated by a cinematographic study. The syncytium is produced by progressive extension of this phenomenon from one cell to the other. Each syncytial area can be considered as resulting from one infectious unit.

CA virus produces giant cells and intracytoplasmic inclusion bodies in monkey kidney tissue culture. These are eosinophilic when stained by hematoxylin and eosin; they are irregularly shaped, granular in structure, morphologically related and closely similar to the intracytoplasmic inclusions produced by measles. The chemical composition of these inclusions was investigated by cytochemical methods. There is good evidence that they contain a high concentration of RNA and SH groups. The question arises as to whether these cytological changes are to be considered as specific characteristics of myxoviruses.

Acknowledgments

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QUANTITATIVE CYTOCHEMICAL INVESTIGATIONS ON THE EFFECT OF VIRUS ON CELLS*

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The effects of viruses on cells have been studied by a great variety of methods, and many contributions to the host cell virus relationship have come from immunology, biochemistry, microscopy, tissue culture, and other disciplines.

One of the problems in which we have been interested for a considerable time concerns the behavior of the nucleoproteins, especially that of the desoxyribonucleic acid (DNA) in cells after viral infection. A fruitful pathway for the study of this particular problem appeared to be the application of quantitative cytochemical techniques such as microspectrophotometry and interference microscopy.[1-4] The development of this new type of microscopy is based largely on the pioneering work of Caspersson,[5] who demonstrated that by extending the optical properties of the microscope into the analytical sphere, the microscope can be used simultaneously as an instrument for both morphologic and chemical analysis of cells. Since the chemical analysis can be done in microscopic preparations *in situ*, that is, without destroying either the architecture of cells or their relationship within a tissue, a direct comparison of cell morphology with chemical composition can be made directly under the microscope.

Although methodological details cannot be discussed here, it should be mentioned that the basic principle of microspectrophotometry, especially when applied to the determination of DNA in single cells, is actually very simple. The Feulgen reaction that leads to a specific staining of the DNA is carried out on microscopical preparations and then, as in the photometric chemical analysis of colored solutions, the amount of light absorbed by the stained cell structure at a wave length characteristic for the DNA dye complex is used as a basis for the qualitative and quantitative analysis of the intracellular DNA.[4]

Thus microspectrophotometry closely resembles analytical chemistry and a microspectrophotometer such as the one that we use in our own laboratories for DNA determination and shown in FIGURE 1 consists actually only of a microscope combined with a device for light absorption measurements.[4] There is a light source *L*, a monochromator or filters *F* to isolate the desired wave length, the microscope *M*, the photometerhead with phototube *PH*, the power supply *P*, and galvanometer *G*; these permit the measurement of the amount of light absorbed by a single cell structure. Light coming only from the cell structure in which the DNA is to be determined is permitted to reach the phototube, the remainder of the light transmitted is obliterated by a diaphragm *D*.

The unique features of this method lie not only in the possibility that a DNA quantity as small as 3×10^{-9} mg can be determined in a well preserved single cell structure,[7,8] but that it is also possible to detect differences in the DNA content from cell to cell that may occur when cells undergo certain changes (be the cause normal or abnormal) such as meiosis, mitosis, degeneration or, as will be discussed in this paper, when cells are infected with virus.

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At present the conventional biochemical methods, in spite of their great contributions to the nucleic acid field do not permit the study of quantitative DNA changes at the single cell level because their analyses must be done on relatively large cell populations where individual cell morphology is destroyed and therefore of necessity the biochemical technique can yield only average values[9, 10]

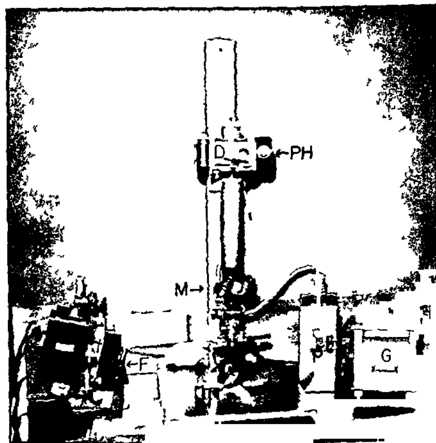


FIGURE 1 Microspectrophotometer

Turning now to the quantitative cytochemical investigations on the effect of viruses on cells it should be pointed out that this paper will deal only with the behavior of the intranuclear DNA in human cell infected with viruses known to contain DNA

In view of the importance of DNA a an essential chemical constituent of cells its great significance for cell life its close relationship to the genes and its quantitative stability in normal cells the study of DNA in cells after they have become infected with a virus which itself contains DNA is obviously of great interest when attempting to elucidate virus host cell relationship Since

the question arose whether the response of the intracellular DNA to the virus DNA is a more or less characteristic one, different types of cells infected with different DNA-containing viruses were studied and two main lines of approach were followed. The first line concerns the DNA behavior of cells *in situ* of tissues from human hosts with virus diseases, the second line concerns the DNA behavior of cells in tissue cultures infected with virus.



FIGURE 2. (*a*) Cells of normal human skin (malpighian layer). Feulgen stain. Approximately ×400. (*b*) Cells of human skin infected with verruca vulgaris. Feulgen stain. Approximately ×400. (*c*) Cells of human skin infected with verruca vulgaris. Feulgen stain. Approximately ×400.

One of the well known virus diseases of the human skin is the common wart verruca vulgaris The characteristic appearance of such infected skin cell under the microscope is seen in FIGURE 2*b* and *c* In contrast to the uninvolved epithelial cells (FIGURE 2*a*), the infected skin cells (FIGURE 2*b* and *c*) show large nuclei, filled with bizarrely shaped, often grapelike masses of DNA-containing material or crystal like structures When nuclear size and DNA content are determined in individual cells of normal skin and compared with those

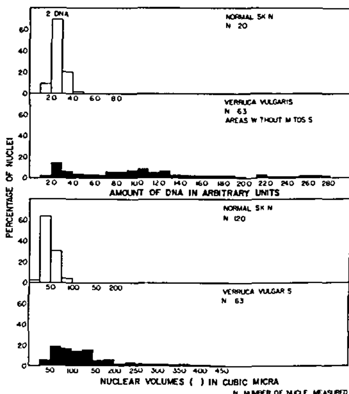


FIGURE 3 Comparison between amounts of DNA (microspectrophotometry) and nuclear sizes of individual cells from normal skin and skin infected with verruca vulgaris

infected with verruca vulgaris data are obtained of which a characteristic example is shown in FIGURE 3 It is evident that the nuclear sizes and the DNA values (as obtained by Feulgen microspectrophotometry) of the normal skin cells show a relatively narrow range that is relatively little variation from cell to cell The mean DNA value corresponds to the characteristic bas

the cells of the skin infected with verruca vulgaris exhibit not only larger and variable nuclear sizes but also strikingly higher DNA values with considerable variability of the DNA content from cell to cell It thus appears that infection

with verruca vulgaris interferes with the stability of the DNA content of the normal skin cells and elicits production of abnormally high amounts of DNA within the nuclei Since these increased quantities of DNA were found in areas of skin without mitosis, the high DNA amounts cannot be explained on the basis of an abnormally rapid cell division, as Leuchtenberger *et al* [11] have reported to be the case in human tumors It is also highly improbable that polyploidy or polyteny may be responsible for these high DNA values because, instead of well-defined multiple groups of 2DNA 4DNA and 8DNA such, as, [illegible]

and *c*)

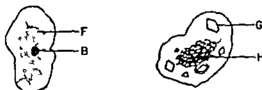
In order to determine whether this abnormal cytological and cytochemical DNA pattern is specific only for skin cells infected with verruca vulgaris in the human host human cells of different tissues infected with different DNA-containing viruses namely adenoviruses were studied in tissue cultures Tissue cultures are very favorable systems for such investigations since the sequence of events in cells can be assessed from a cytological and cytochemical point of view at various known time intervals after the viral infection

In earlier studies by Boyer *et al* [12] HeLa cell tissue cultures were infected with adenoviruses (Types 1 3 and 4) The characteristic stages of nuclear changes in HeLa cells infected with Type 3 or 4 are presented schematically in FIGURE 4 The increase in nuclear size the formation of bizarrely shaped masses of DNA and the occurrence of DNA containing crystal like structures are all features similar to those in verruca vulgaris Quantitative DNA determinations by Feulgen microspectrophotometry also gave higher DNA values for the HeLa cells infected with adenovirus than for the uninfected HeLa cells However HeLa cells are not a very suitable cell type for comparative quantitative DNA studies This is due to the fact that the DNA values in the interphase nuclei of the noninfected HeLa cell control culture show relatively high and variable amounts (their basic value being 4DNA) from cell to cell which of course is not surprising since we deal with cancer cells undergoing rapid often abnormal divisions Therefore, it seemed desirable to use another human cell type preferably a normal cell with a basic diploid DNA content and with a relatively slow mitotic process Amniotic tissue cultures which we prepared according to the technique of Dunnebacke and Zitcer [13] were very favorable material for such studies The cytological features of uninfected amniotic [illegible]

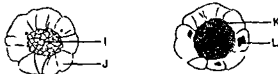
was infected with the adenovirus The characteristic DNA values of the interphase nuclei of different periods after infection are presented in FIGURE 6 In order to establish a base line, the DNA values were determined first in sections of the amnion fixed immediately *after* it had been obtained from the



STAGE 1



STAGE 2



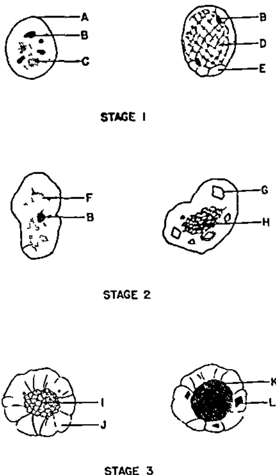
STAGE 3

delivery and *before* it was trypsinized and used for tissue culture It is evident that the amnion contains cells with a basic 2DNA content, but that there are also a few cells with higher DNA values, which are between two and four times the basic values A picture similar to that of the amnion sections is obtained when the DNA content was determined in the first generation tissue cultures of the same amnion, here again cells were found with DNA contents between



FIGURE 5 (*a*) Amniotic cells of control cultures (first generation amnion No 5) Feulgen stain Approximately ×400 (*b*) Amniotic cells 72 hours after infection with adenovirus Type [illegible] Feulgen stain Approximately ×400 (*c*) Amniotic cells 96 hours after infection with adenovirus Type 7 Feulgen stain Approximately ×400

was infected with the adenovirus The characteristic DNA values of the interphase nuclei of different periods after infection are presented in FIGURE 6 In order to establish a base line, the DNA values were determined first in sections of the *amnion fixed immediately after it had been obtained from the*



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delivery and *before* it was trypsinized and used for tissue culture It is evident that the amnion contains cells with a basic 2DNA content, but that there are also a few cells with higher DNA values, which are between two and four times the basic values A picture similar to that of the amnion sections is obtained when the DNA content was determined in the first generation tissue cultures of the same amnion, here again cells were found with DNA contents between



FIGURE 5 (*a*) Amniotic cells of control cultures (first generation amnion No 5) Feulgen stain Approximately ×400 (*b*) Amniotic cells 72 hours after infection with adenovirus Type 7 Feulgen stain Approximately ×400 (*c*) Amniotic cells 96 hours after infection with adenovirus Type 7 Feulgen stain Approximately ×400

2DNA 4DNA, and 8DNA Whether the DNA values that are higher than 4DNA are due to synthesis of the diploid DNA cells or whether they are due to the fact that the amnion contains a few tetraploid cells that synthesize DNA cannot be decided at present, since mitotic figures were so scant that chromosomal counts could not be made The possibility that the amnion does contain polyploid or polytene cells, that is, nuclei with 2DNA and 4DNA content must be considered seriously, since we know that *normal* tissues

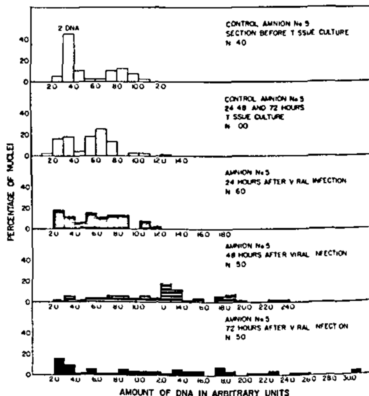


FIGURE 6 Comparison between amounts of DNA (microspectrophotometry) in individual nuclei of human amniotic cells before and after infection with adenovirus Type 7

made up of only diploid that is 2DNA cells, even when undergoing rapid mitosis, will show cells with a DNA content between 2DNA and 4DNA only and not higher

Perhaps the presence of 4DNA, that is tetraploid cells in the normal amnion *in situ* explains the frequently reported heteroploid transformation in amniotic tissue cultures[14] Since tetraploid cells seem to have a selective advantage in tissue cultures,[15] the heteroploid cells may actually derive from the tetraploid ones

Comparing the DNA data from the noninfected control amniotic cells with those found at varying time intervals after the viral infection it can be seen

that 24 hours after infection with adenovirus Type 7, the DNA values are essentially the same as those of the noninfected controls However 48 and 72 hours after viral infection the DNA values are considerably higher with a pronounced variability from cell to cell, resembling closely the DNA values in verruca vulgaris (FIGURE 3) Essentially the same pattern was found in other experiments in which we used amnions from other human deliveries and where the DNA values were determined up to 96 hours after viral infection

On the basis of the few examples presented here, it appears that when human cells are infected with certain DNA-containing viruses such as verruca vulgaris or adenoviruses, characteristic cytological and cytochemical changes involving the DNA occur in the nuclei of the infected cells The cytological response and the deviating DNA pattern were found to be very similar in the cases examined irrespective of whether the cells infected were skin cells, amniotic cells, or cancer cells and regardless of whether the cells were still *in situ* in tissues of the human body or were explanted to grow in tissue culture

Although these data clearly demonstrate the fact that these viruses elicit an abnormal DNA metabolism in the nuclei and disrupt the DNA constancy, the interpretation of the deviating DNA behavior can be only speculative One of the great difficulties, when attempting to explain the abnormally high DNA values comes from the impossibility of differentiating *in situ* between the intranuclear virus DNA and the host cell DNA by the present methods, including the Feulgen reaction Therefore at present it cannot be decided whether the large DNA amounts observed are due to multiplying virus DNA or due to an unusual and irregular synthesis of host cell DNA induced by the presence of the virus That the changes observed may reflect viral propagation is suggested by the similarity of the cytological and cytochemical behavior of cells infected with other viruses such as herpes simplex[18] and the polyhedra virus of the silkworm[17] in which intranuclear viral synthesis is believed to occur Furthermore the character of these intranuclear changes seems to be almost specific for these virus infected cells and is strikingly different from the appearance of the nuclei of cells engaged in growth mitosis or other metabolic activities and from cells undergoing necrosis and pyknosis[18] As a matter of fact when the microscopist is confronted with the peculiar combination of intranuclear bizarre configuration of DNA masses associated with increase and variability of DNA which cannot be explained on the basis of mitosis polyploidy or polyteny viral etiology should be considered seriously Moritz and Leuchtenberger reported such a case in a young man who died of an unexplained respiratory disease[19] Nearly all the tissues examined contained cells with large nuclei and intranuclear alterations very similar to those found in the virus infected cells just described In FIGURE 7 the nuclear sizes and the DNA values in the kidney of this man as compared with the values of a normal human kidney are graphed The similarity of the DNA pattern in the kidney nuclei of this case with those of the verruca or adenovirus infected nuclei is evident Although we realize of course that the data are not final proof for establishing a viral origin the concept of a possible viral infestation was offered with the suggestion that other pathological tissue processes of unknown etiology presenting similar findings also would be examined from a viral point of view

Summarizing briefly, human cells infected with certain DNA-containing viruses, such as verruca vulgaris or adenovirus, reveal characteristic cytological and cytochemical abnormalities of the DNA pattern. This deviating behavior of the DNA was observed regardless of the type of cells infected (skin, cancer amnion) and irrespective of whether the cells were *in situ* in tissues of the human body or whether they were explanted to grow in tissue culture. The similar response of the cells is especially encouraging, because tissue cultures are

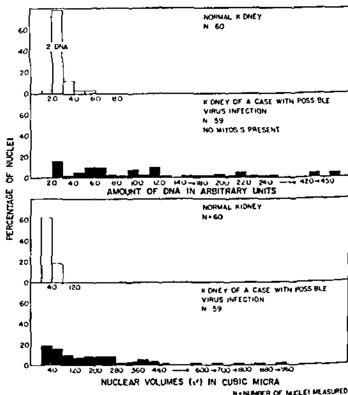


FIGURE 7. Comparison between amounts of DNA (microspectrophotometry) and nuclear sizes of individual cells from normal kidney and kidney from a patient with possible systemic virus disease.

excellent biological systems for the correlated cytological and cytochemical exploration of the sequential changes occurring in cells after viral infestation. It is hoped that this brief presentation on the quantitative cytochemical investigation of the DNA in virus-infected cells demonstrates the new pathways opened by these cytochemical procedures and stimulates the application of these processes to the elucidation of the many unexplored problems concerning the virus-host cell relationship.

Acknowledgment

We gratefully acknowledge the valuable technical assistance of Gergin Gelev

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FLUORESCENCE MICROSCOPY OF NUCLEIC ACID CHANGES IN VIRUS INFECTED CELLS

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In the past few years, chemical analysis of purified virus preparations has revealed with increasing certainty that the essential polynucleotide component may be either desoxyribonucleic (DNA) or ribonucleic acid (RNA) It is now established that influenza poliomyelitis, eastern equine encephalomyelitis and West Nile encephalomyelitis viruses contain RNA, vaccinia, Shope papilloma and bacteriophage contain DNA An investigation of the changes in nucleic acid distribution in virus infected cells thus might be expected to yield information concerning the intracellular site of virus formation and help to elucidate the cytological appearances that are of constant occurrence in some virus infections During a study of the cytochemical significance of the tissue fluorescence induced by the fluorochrome, acridine orange, Armstrong (1956) found that in thin sections of fixed tissues treated with a dilute solution of the dye, it was possible, with the fluorescence microscope, easily to locate the distribution of nucleic acid-containing material within tissue cells and to distinguish between RNA and DNA A similar conclusion was reached by Bertalanffy and Bickis, 1956 The advantages of the technique are its simplicity and sensitivity as compared with established methods of staining nucleic acid material and the simultaneous demonstration of both RNA and DNA by a single solution The sensitivity is particularly well brought out in monolayer tissue culture preparations

Although all the acridine compounds possess the property of fluorescence the aminoacridines exhibit this phenomenon to a remarkable degree The brilliant polychromatic fluorescence shown by acridine orange was described by Strugger (1940) who was the first to apply a method of induced fluorescence to the investigation of microbiological problems Using acridine orange on unfixed material, he claimed to be able to distinguish between living and dead bacterial and plant cells The differential fluorescence that can be obtained on unfixed material has been interpreted variously by subsequent workers and there is still no unanimity of opinion concerning the results, at the present time the value of the technique for determining the viability of cells has not been established Investigation of the chemotherapeutic properties of the amino acridines has indicated that acridine orange and some related compounds have a marked affinity for nucleic acid-containing structures both *in vivo* and *in vitro*, and this specific interaction forms the basis of the technique that has been used for observations on virus infected cells (Armstrong and Niven 1957) Anderson (1957) using a similar technique, has studied the multiplication of bacteriophage in lysogenic bacterial cells induced with ultraviolet radiation

Since maximal activation of the fluorescence occurs in the blue violet region of the spectrum, quartz optical components are not required, a 250 watt high pressure mercury vapor lamp provides an adequate light source and with a dark ground condenser and suitable filtration, the fluorescent images stand out clearly against a black background In 3 μ sections of fixed animal tissues

structures containing DNA such as nuclear chromatin, show a greenish yellow fluorescence while RNA-containing material gives a bright flame red color. The procedure can be applied equally to smears of cells and tissue culture cells grown on cover slips. Fixation is not critical, only osmium tetroxide and fixatives such as Bouin's fluid containing picric acid inhibit the specific fluorescence. The most important controlling factor is the pH of the staining solution. Within a pH range of 1.5 to 3.5 for acridine orange or 3.5 to 5.0 for acridine orange R, the 5-phenyl derivative, differential fluorescence is most striking. The findings have been confirmed by parallel studies of similar material using the Feulgen reaction, methyl green pyronine staining, nuclease digestion tests and ultraviolet absorption techniques. Since the tissues and cells have been subjected to histological fixation the experimental material is always accompanied by appropriate uninfected controls and, where possible, the microscopic observations have been correlated with virus titration of the material examined. Within the pH range mentioned previously only structures containing acid mucopolysaccharides such as mast cell granules, cartilage and elastic tissue exhibit fluorescent properties at all similar to those of nucleic acid-containing elements, but identification of these presents no problem.

Members of the pox group of viruses provided interesting material for study. In the case of vaccinia the Guarnieri bodies in corneal epithelium, which are known to be Feulgen positive, gave a positive DNA fluorescence with the acridine orange technique. In ectromelia (mouse pox) the eosinophilic diagnostic inclusions in squamous epithelium produced by the virus gave a negative result with acridine orange as did the similar inclusions occasionally found in the pancreas and the intestinal epithelium; they also gave a negative Feulgen reaction and did not stain with either pyronine or methyl green. Since early work by Barnard and Elford (1931) had indicated that these inclusions contain virus elementary bodies, it is obvious that this problem requires further study. Extensive necrosis occurs in the liver during the course of the disease but although virus can be recovered in high titer from this organ, inclusions identical with those of the squamous epithelium do not occur. Many cells, however, were found to contain spherical bodies showing the typical greenish yellow DNA fluorescence. In cells containing such bodies the nuclei, apart from enlargement and increased prominence and hypertrophy of the nucleoli, were at first apparently normal. The RNA fluorescence of the cytoplasm gradually diminished and eventually disappeared as the cytoplasmic DNA bodies increased in size. Treatment of such sections with 0.01 per cent (w/v) desoxyribonuclease (DNase) at pH 7.4 removed the fluorescence of normal nuclear DNA but did not interfere with the fluorescence of the cytoplasmic DNA in infected cells. Pretreatment with 0.02 per cent pepsin at pH 2.0 for 2 hours at 37° C, which did not inhibit the specific DNA fluorescence, rendered the cytoplasmic DNA susceptible to subsequent digestion by DNase.

Bearcroft and Jamieson (1958) have recently described an infectious disease of rhesus monkeys, transmissible by filtered suspensions, which occurred at Yaba, Lagos, Nigeria. The disease is characterized by subcutaneous tumorlike lesions that persist for periods up to 10 weeks and then regress. Smears from the cells composing the nodules show the presence of numerous elementary bodies that give a light greenish yellow fluorescence with acridine orange and,

when examined with the electron microscope, are seen to resemble vaccinia in shape and size and in the possession of a pepsin resistant central dense body. The characteristic cells apparently derived from fibroblasts are polygonal in shape with large vesicular nuclei and greatly hypertrophied nucleoli. The cytoplasm contains dense paranuclear bodies of varying shape and size, often coexisting with areas of fine granularity. All these abnormal cytoplasmic structures exhibit a bright DNA fluorescence, and the appearances suggest a series of developmental stages, the earliest being the dense body that becomes transformed into granular material. As in the case of liver cells infected with ectromelia pretreatment with pepsin was necessary before the cytoplasmic DNA could be removed with DNase.

Observations by conventional light microscopy have disclosed profound alterations of nuclear structure in HeLa cells infected with adenoviruses (Barski 1956; Boyer *et al.* 1957). By combining electron microscopy of ultrathin sections with the Feulgen reaction in alternate thin and thick sections, Bloch *et al.* (1957) have localized and determined that the aggregations of virus particles correspond to the Feulgen positive intranuclear material and they consider the virus to be of DNA type. Armstrong and Hopper (in press) studied the cytochemical changes in the HLp 2 line of human cells after infection with adenovirus Type 6 with the acridine orange fluorescence technique and found that enlargement of the nucleolus accompanied by enhanced fluorescence was the earliest detectable change; this was soon followed, however, by changes in the pattern of nuclear chromatin. Irregular masses of intranuclear material showing an intense fluorescence of the DNA type associated with a system of vesicles within the nuclear membrane developed, the masses frequently fusing to form a centrally placed body within which the red fluorescing nucleolus could still be made out. This intranuclear DNA is not directly susceptible to the action of DNase but can be removed by the specific enzyme after treatment with pepsin. It thus possesses, like the cytoplasmic DNA already described in other virus infected cells, a protective component that can be removed by proteolytic digestion.

The cytopathogenic changes that occur in tissue culture cells infected with the virus of cytomegalic inclusion disease have been described by Weller *et al.* (1957). Through the kindness of Weller it has been possible to make some observations on the development of the intranuclear inclusion bodies that are so characteristic of this condition. Enlargement of the nucleus, hypertrophy and increased intensity of fluorescence of the nucleoli, and the appearance of numerous bright greenish yellow fluorescing granules were the earliest changes detected. In all cases the RNA fluorescence of the cytoplasm also increased in intensity particularly around the nucleus. The typical intranuclear inclusion body is formed apparently by the enlargement and fusion of the numerous granules of DNA material seen at an earlier stage. Nuclear vacuolation so characteristic of adenovirus infection does not take place.

The cytopathological effects of West Nile encephalitis have been studied in monolayer cultures of chick fibroblasts. This virus is known to contain RNA as its essential polynucleotide component (Colter *et al.* 1957); it was of interest to see if the usual pattern of RNA was changed during virus growth. Enlargement of the nucleus and increased prominence of the nucleolus followed infec-

tion but these reactions were not accompanied by significant alterations of nuclear morphology. The major site of cytological change was in the cytoplasm where the fine RNA granularity was replaced by brighter and coarser RNA fluorescence. Conspicuous masses of bright red material appeared, subsequently, these could be seen close to the cell surfaces attached to long tenuous processes or lying free. As these cytoplasmic aggregates accumulated nucleolar and nuclear fluorescence gradually diminished. The study of influenza infected tissue cultures has proved to be one of the most interesting applications of the acridine orange technique. Enlargement of the nucleus and of the nucleolus was accompanied by the development of a diffuse red color that filled the nucleus often obscuring all the nuclear structures except the associated chromatin of the nucleoli. The sharp outline of the nuclear membrane became indistinct and acquired a fine beaded appearance suggestive of breaks in continuity. In the cytoplasm, an increased intensity of RNA fluorescence frequently was followed by the development of intense perinuclear fluorescence or by the appearance of tufts of flame red color extending from one or both nuclear poles. The maximal intensity of nuclear fluorescence however was not necessarily accompanied by the most striking cytoplasmic changes. The intranuclear RNA fluorescence was removed by RNase, pretreatment with a proteolytic enzyme was not required.

Summary

In these studies of virus infected cells certain features have been found some of which recall alterations that can occur in physiological conditions and others that so far as is known are not associated with normal cytological activity. The former are illustrated by nuclear and nucleolar enlargement and an increase in cytoplasmic RNA, the latter by qualitative and quantitative changes in the distribution of nucleic acids within the nucleus or cytoplasm or both. It would appear that unique changes in nucleic acid pattern can be initiated following virus infection and these are of 4 kinds (1) appearance of DNA in the *cytoplasm* as in pox virus infections and the spontaneous disease of rhesus monkeys which is being investigated, (2) an altered RNA pattern in the cytoplasm as in West Nile encephalitis, (3) the development of RNA in the nucleus quite distinct from the nucleolus as in influenza and (4) the occurrence of DNA aggregates in the nucleus as in adenovirus infections and cytomegalic disease of infants. It may be significant that in all cases where the nucleic acid constituent of a virus agent is known the dominant change has occurred in that nucleic acid type.

It can be argued of course that the changes recorded may be the result of disordered cell metabolism following infection and there is certainly no justification for assuming on the basis of such observations alone that any unusually sited nucleic acid is virus nucleic acid that will be incorporated in fully developed infective particles unless the results are supported by collateral evidence from electron microscopy and immunological data such as is given by the Coons fluorescent antibody technique. In the case of DNA viruses, however, we have found a relative insusceptibility of the abnormal DNA to the action of DNase in all the material so far examined and subsequent work may reveal some distinguishing quality of the abnormal RNA in cells infected with

RNA viruses It is necessary to study more viruses, and more information may become available by studying the effect of individual viruses in different types of host cells The method thus may be capable of giving a hint of the nucleic acid structure of a particular virus

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CELLULAR CHANGES ASSOCIATED WITH INFECTION OF THE EHRLICH ASCITES TUMOR WITH NEWCASTLE DISEASE VIRUS*

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The interaction of Newcastle disease virus (NDV) with Ehrlich ascites tumor (EAT) cells of the mouse has been the subject of several reports published within the past few years.[1-4] These studies have employed NDV adapted to growth in EAT cells, referred to as EAT-adapted NDV, as well as ordinary laboratory strains propagated by serial passage in eggs, referred to as chick embryo-adapted NDV. The EAT-adapted virus is able to replicate infectious virus in EAT cells,[1,2] while chick embryo-adapted NDV is not,[3,4] although the latter is able to infect and destroy EAT cells. This paper is limited to a consideration of the morphology of Ehrlich ascites tumor cells infected with chick embryo-adapted Newcastle disease virus.

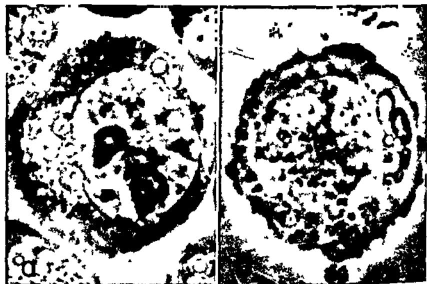
In a recent study of the interaction of chick embryo-adapted NDV with EAT cells, the fluorescent antibody technique was employed to demonstrate what appeared to be an exceptionally rudimentary form of incomplete virus.[4] The results of this study indicated that infection of EAT cells with this virus resulted in the intracytoplasmic synthesis of a component that had the ability to combine specifically with fluorescein-labeled anti-NDV serum but that had no detectable hemagglutinin activity and no measurable complement-fixing ability, and was not infective for embryonated eggs. This observation suggested the interesting possibility that the newly synthesized component might be only a small fragment of the intact virus particle, possibly a single molecular species. Replication of a more complex form of virus was by no means ruled out, however; for this reason, morphologic study of the Ehrlich-NDV system was carried out in an attempt to determine whether the newly synthesized antigen was as simple morphologically as it was functionally, or whether it was associated with a unit more nearly resembling the intact infective virus, as is the case with "incomplete" influenza virus produced by passage in the mouse brain or by serial undiluted passage in the allantoic cavity of the chick embryo.[7,8]

Our approach was threefold and employed the following procedures: phase microscopy carried out on fresh unfixed wet preparation; conventional light microscopy carried out on paraffin-embedded cells stained with hematoxylin and eosin, Giemsa, pyronine-methyl green, Feulgen, and methenamine silver; and electron microscopy of material that had been fixed in osmium tetroxide and embedded in methacrylate. A detailed description of materials and techniques employed has been published elsewhere.[9]

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Phase Microscopy and Light Microscopy

A typical infection experiment as observed with the phase microscope occupied a total of less than 15 hours Washed Ehrlich ascites cells, harvested 6 days after inoculation were sedimented by low speed centrifugation and resuspended in a partially purified preparation of NDV The concentration of virus and cells was adjusted to give a ratio of from 50 to 1000 infective particles per cell The mixture of virus and cells was then placed in an ice bath for about 30 min in order to allow adsorption to take place Following adsorption aliquots of the mixture were injected into the peritoneal cavity of



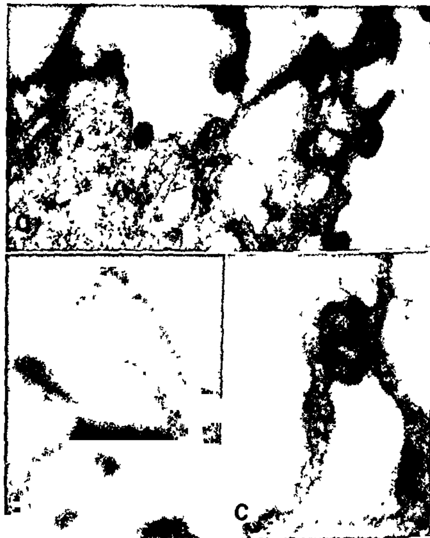
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fresh mice and the infection allowed to proceed, samples being removed at microscope typical uninfected fine hairlike pseudopodia averaging about 1 μ in length a well-defined nucleus with irregularly clumped chromatin and two readily distinguishable cytoplasmic components mitochondria that appear as short dark rodlets and brilliantly refractile objects that have been demonstrated to contain lipid Infected cells, in the time interval 0 to 4 ... from the normal cell show ... infection a new cla... objects gradually increase in size and number until virtually every cell ... infected population is involved Initially, they usually occupy a juxtanuclear

position and are neither as dark as mitochondria nor as refractile as lipid droplets. Their contours are usually smoothly lobulated and little internal structure can be visualized at the resolution offered by the phase microscope. Numerous variations in shape are usually seen. Some are approximately spherical and measure from 1 to 4 μ in diameter. Still others are ellipsoidal, and occasional forms are greatly elongated measuring up to 10 μ or more in length. The ultimate fate of infected cells is lysis. With the virus cell ratios employed in this experiment the lytic process begins 6 to 8 hours following infection; usually by 12 to 15 hours the vast majority of the infected cells has undergone disintegration. The nuclei of the disintegrating cells usually showed little change until after complete dispersion of the cytoplasmic components when a gradual process of pyknosis set in, ultimately resulting in small dark irregular masses of nuclear material about the size of a red blood cell. The process of disintegration as viewed with the phase microscope was never a sudden fulminant event but usually occupied an interval of 15 to 30 min. The extended duration of the dissolution process seemed to be due primarily to the presence in the vicinity of the nucleus of an invisible but highly viscous gel that hindered the centrifugal motion of the cytoplasmic material. Conventional light microscopy performed on paraffin embedded material failed to reveal clear-cut evidence of the cytoplasmic bodies demonstrated so clearly with phase contrast.

Electron Microscopy

Cells examined after adsorption of virus but prior to placement in the peritoneal cavity of the mouse differ morphologically from control cells in only one particular: adsorbed particles tentatively identified as Newcastle disease virus may be seen on and between the pseudopodia (FIGURE 2a, b and c). The over all morphology of these particles is quite uniform, although there is some variation in size. These particles can be seen to have a double limiting membrane each element of which measures approximately 30 to 40 Å in thickness enclosing several small dense regions that are rather uniform in size and measure about 110 Å in diameter. The identification of these particles as Newcastle disease virus is supported by several considerations. First, they are denser and more uniform and have a more complex structure than do cross sections of pseudopodia with which they might be confused. Second, their measurements agree roughly, assuming a spherical shape, with other estimates of the size of NDV. Third, similar particles have been identified in electron micrographs of NDV agglutinated chicken red blood cells but have never been seen on uninfected Ehrlich cells or on chicken red blood cells suspended either in various buffers or in uninfected chick embryo allantoic fluid. Finally, the particles disappear from the surface of Ehrlich cells within 30 min after infected cells are placed in the peritoneal cavity of the mouse. This is in agreement with fluorescent antibody studies which indicate that virus adsorbed to the surface of EAT cells is initially visible as a bright peripheral ring of fluorescence that disappears after incubation for 15 to 30 min either *in vivo* or *in vitro*.[6] Disappearance is not due to elution, at least of hemagglutinating particles. The mode of penetration of the plasma membrane by the virus



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... A hint of what may be occurring, however, ... which there ... is particle at ... adjacent cell wall. Substantiation of this point would suggest that the inner portion of the virus may enter the cell prior to, and perhaps separate from, the outer coat of

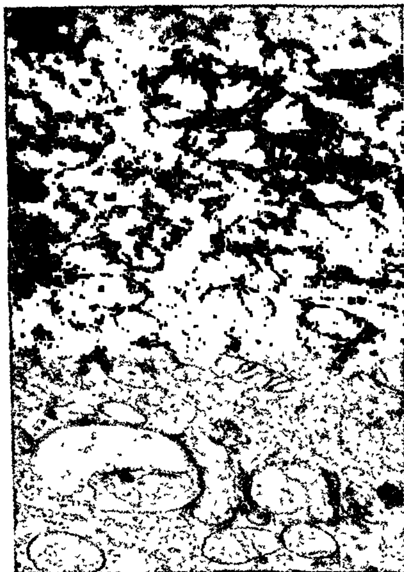


FIGURE 3 Electron micrograph of a portion of the cytoplasm of an Ehrlich ascites tumor

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the virus particle Cells studied at intervals of 30 min and 2 hours following infection no longer have the adsorbed surface particles just mentioned and are indistinguishable from control cells The cytoplasmic inclusions that appear following infection with Newcastle disease virus are first clearly seen after 4 hours incubation in the peritoneal cavity of the mouse (FIGURE 3) There



after they increase rapidly in size and number, as FIGURE 4, 10½ hours after infection, illustrates. No nuclear involvement has been seen in any of the preparations studied. Although the inclusions exhibit individual variations in appearance, their over-all morphology has several characteristic features



FIGURE 5. Electron micrograph of an Ehrlich ascites tumor cell 10½ hours after infection with NDV illustrating the close association between Golgi material and the shell of the inclusions. ×39 000. Reproduced by permission from *The Journal of Experimental Medicine*.[9]

illustrated in FIGURES 5 and 6. Usually they are elliptical or circular in profile and may measure up to several microns in diameter. They have a limiting shell composed of a variable number of laminae measuring 4 to 8 mμ in width and usually associated with small vesicles. The limiting shell encloses a core



FIGURE 6 Electron micrograph of an inclusion adjacent to the nucleus of an Ehrlich as-

later for example at 8 or 10 hours after infection usually had at least a dozen layers and occasionally many more The vesicles associated with the laminae have the appearance of transversely sectioned tubules that resemble and are frequently continuous with adjacent clusters of vesicles that can be identified as elements of the smooth surfaced reticulum or Golgi material Frequently 1 of the laminae can be seen to split into 2 thinner lamellae that are continuous



FIGURE 7. Electron micrograph of an Ehrlich ascites tumor cell 10½ hours after infection with Newcastle disease virus. At this stage of infection approximately 5 to 10 per cent of infected cells revealed varying degrees of vacuolization and mitochondrial swelling as depicted in this figure. This cell is identified as an EAT cell by the presence of two characteristic intrareticulum particles of EAT at the extreme upper left (arrow). ×22,500. Reproduced by permission from *The Journal of Experimental Medicine*.[8]

with the wall of 1 of the adjacent vesicles. An example of this may be seen at the bottom of FIGURE 5. This observation strongly suggests that the laminar portion of the shell may be formed by a process of collapse and fusion of vesicles of the smooth surfaced reticulum. Considerable variation in the morphology of the internal granular material was noted among different inclusions; however, the granules within any particular inclusion displayed a somewhat greater uniformity. No correlation was demonstrated between the

size of the granules and the size of the inclusion, or the number of laminae comprising its shell. Few if any significant changes were seen in the cytoplasmic organelles during the early stages of infection. However, in the end stages of infection, marked vacuolization of the vesicular components of the

numbers of them were seen extracellularly at a time when cytolysis was progressing rapidly.

Comments

In answer to the initial question concerning the morphology of the incomplete viral antigen previously demonstrated by fluorescent antibody studies, these results would seem to suggest strongly that the newly synthesized antigenic units are much smaller than the native virus particles, perhaps no larger than 10 to 14 mμ in diameter, and that they may, in fact, be identical with the granular material present in the core of the cytoplasmic inclusions. This antigenic material has been thought of as a subunit of the intact virus. However, the possibility that the antigen represents a nonviral, noncellular protein associated with the infection process must also be considered. Any hypothesis linking the granules that comprise the core of the inclusions with the fluorescent antibody binding antigen is, of course, highly conjectural, particularly since t'

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to be tested further. First, no large particles bearing resemblance to the native virus particles were ever seen in any of the sections examined. Second, the particles within the inclusions exhibit much greater variation in size than do the cytoplasmic granules. Third, no forms demonstrating continuity between the granular material of the inclusion and the cytoplasm of the cell have been seen among the many hundreds of inclusions studied in the electron microscope. Finally, it is not unreasonable to assume that the newly formed antigenic units, if they are visible at all, most likely would be associated with the newly formed morphologic entity, that is, the cytoplasmic inclusion. Another possibility, of course, is that the new synthesized antigenic material is associated with the shell of the inclusion rather than with the granular material in its core. This seems improbable, however, in view of the demonstrated close association of the shell with the Golgi material of the Ehrlich cell, suggesting that the shell is of cytoplasmic origin.

The general significance of the cytoplasmic inclusions here demonstrated remains speculative. Even though these inclusions seemed to be unquestionably related, in this particular system, to infection of cell with virus, it is unlikely that they are unique to this particular cell system for two reasons. First, although none of the control preparations in the present set of experiments contained structures bearing any resemblance to the inclusions that developed in infected cells, such structures have been seen on rare occasions in our laboratory in other preparations of uninfected Ehrlich cells. Second, similar

appearing laminated structures have been described by other investigators in other virus-cell systems.[19] These considerations suggest the possibility that this type of inclusion is simply a particular example of a more generalized reactive phenomenon on the part of Ehrlich ascites cells and possibly of other cell types as well. If this is a valid generalization, one might then envision a new and unsuspected function of the Golgi apparatus, that is, participation in a type of intracellular defense mechanism. The possibility of such a function is certainly reasonable on theoretical grounds and receives some additional support from recent and rapidly developing evidence that the physiological state of the host cell is of paramount importance in determining not only whether infection occurs but to what extent the invading virus is successful in commandeering intracellular systems for the purposes of self duplication. Two examples that lend support to this idea are, first, a study of NDV infected L cells by Wilcox[11] which demonstrated the fact that the quantity of infective virus produced by infected cells can be increased by injuring the cells (either by decreasing incubation temperature or by treating them with sublethal concentrations of cyanide) and, second, the observation that chick embryo-adapted NDV is able to undergo complete replication in Ehrlich ascites tumor cells only in tissue culture under suboptimal nutritional conditions.[1,2] It remains to be seen whether the presence or activity of the laminated inclusion plays any role in those functions of the cell that are able to limit the process of virus multiplication to some form of partial replication.

Summary

The morphologic changes occurring in Ehrlich ascites tumor cells infected with chick embryo adapted Newcastle disease virus were studied with phase microscope, conventional light microscope and electron microscope. Intracytoplasmic inclusions appeared 2 to 4 hours following infection and progressively increased in size and number until cytolysis occurred. Significant alterations in mitochondria and other cell organelles did not appear during the initial period of inclusion development but were seen shortly before cytolysis began (8 to 10 hours after infection). The inclusions were composed of a laminated shell possibly derived from the agranular reticulum of the EAT cell, surrounding a central portion containing dense particles that measured 3 to 14 mμ in diameter. The identity of these particles and a possible function of the inclusions are discussed.

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size of the granules and the size of the inclusion, or the number of laminae comprising its shell. Few if any significant changes were seen in the cytoplasmic organelles during the early stages of infection. However, in the end stages of infection, marked vacuolization of the vesicular components of the cytoplasm occurred and at the same time, the cytoplasmic inclusions appeared to approach the plasma membrane (FIGURE 7). The inclusions appeared to be at least somewhat resistant to the process of dissolution since increasing numbers of them were seen extracellularly at a time when cytolysis was progressing rapidly.

Comments

In answer to the initial question concerning the morphology of the incomplete viral antigen previously demonstrated by fluorescent antibody studies, these results would seem to suggest strongly that the newly synthesized antigenic units are much smaller than the native virus particles, perhaps no larger than 10 to 14 mμ in diameter, and that they may, in fact, be identical with the granular material present in the core of the cytoplasmic inclusions. This antigenic material has been thought of as a subunit of the intact virus. However, the possibility that the antigen represents a nonviral, noncellular protein associated with the infection process must also be considered. Any hypothesis linking the granules that comprise the core of the inclusions with the fluorescent antibody binding antigen is, of course, highly conjectural particularly since these particles are within the size range of the RNA-containing cytoplasmic granules and may represent nothing more than isolated islands of cytoplasm. Several considerations, however, do recommend this as a working hypothesis to be tested further. First, no large particles bearing resemblance to the native virus particles were ever seen in any of the sections examined. Second, the particles within the inclusions exhibit much greater variation in size than do the cytoplasmic granules. Third, no forms demonstrating continuity between the granular material of the inclusion and the cytoplasm of the cell have been seen among the many hundreds of inclusions studied in the electron microscope. Finally, it is not unreasonable to assume that the newly formed antigenic units, if they are visible at all, most likely would be associated with the newly formed morphologic entity, that is, the cytoplasmic inclusion. Another possibility, of course, is that the new synthesized antigenic material is associated with the shell of the inclusion rather than with the granular material in its core. This seems improbable, however, in view of the demonstrated close association of the shell with the Golgi material of the Ehrlich cell, suggesting that the shell is of cytoplasmic origin.

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CYTOPATHOLOGY OF VIRUS INFECTED TUMOR CELLS

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Introduction

Thirty six years ago Levaditi and Nicolau demonstrated the multiplication of herpes simplex and neurovaccinia viruses in transplantable carcinomata of mice[1,2] Growth of neurovaccinia virus was accompanied by oncolysis[3] Since then the oncolytic effect of virus infection has been demonstrated repeatedly[4,5] The possible value of viruses in the treatment of cancer and, in particular, of viruses adapted to grow in tumors has not been assessed fully The disappointing results of chemotherapy[6] and, by comparison, the remarkable oncolytic action of many viruses[4,5,7-10] would seem to justify further research in this field

The implication of viruses in the initiation, promotion, and causation of some forms of neoplasia is well known Latent virus infection in tumors has been suspected[11] and demonstrated[14] Thus, apart from the need to acquire fundamental knowledge of the virus-cell relationship, the study of virus-infected tumor cells may facilitate an understanding of the relationship between virus infection and neoplasia

Certain patterns of cytopathology become apparent in the study of different types of tumor cells infected with viruses Some of these patterns simulate closely disorders of the cell resulting from other causes In discussing the cytopathology of various virus infections an attempt will be made to compare and contrast cellular degeneration that is not attributable to known virus infection

Cytochemistry of Uninfected Ehrlich Ascites Tumor Cells

The morphology of an uninfected Ehrlich ascites tumor stained by a modified bone marrow strain[15] is shown in FIGURE 1 The nucleus contains several prominent nucleoli, inside many of which there are fine vacuoles In addition to the chromatin and nucleoli, the nucleus also contains amorphous, finely granular parachromatin[16] (or nuclear "sap"), which is acidophilic in this preparation In old tumors, such as the one shown in FIGURE 1, the chromatin and the parachromatin of an occasional cell are marginated and one or more acidophilic bodies or inclusions* are formed The nature of these bodies can be elucidated in preparations stained by the toluidine blue molybdate (TBM) method[17] FIGURES 2*a*, *b*, *c*, and 3*a* It may be emphasized that all the staining in FIGURES 2*a*, *b*, *c*, and 3*a* is due to ribonucleoprotein (RNA protein) In preparations stained by the TBM method, the RNA protein of the parachromatin is stained purple and that of the nucleolus, green Many of the nucleoli contain metachromatic RNA protein bodies or nucleolini,[16] as shown in FIGURE 2*b* The nucleolini appear to correspond to the vacuoles seen in other preparations (compare this with FIGURE 1) A cell showing margination of the parachromatin with the formation of inclusions containing RNA is shown in FIGURE 2*c*

* The term inclusion or inclusion body is used throughout this paper to refer to something other than the normal organelles, that is included in the cell and that need not be a *de novo* formation



FIGURE 1 Smear of Ehrlich ascites tumor stained by a modification of Barrett stain[18] Note the minute vacuoles (V) in a nucleolus Two acidophilic bodies (B) are present in a degenerating cell ×1600



Under the microscope it may be seen that the large inclusions consist of a homogeneous green staining center, derived from the nucleolus, covered by purple parachromatin granules In other degenerating cells smaller inclusions appear to be entirely formed by condensation of the parachromatin (FIGURE 3*a*) A study of old ascites tumors reveals that these cells eventually disintegrate by karyorrhexis and that the fragments are ingested by macrophages

The cytoplasm of the Ehrlich ascites tumor cell contains three kinds of lipid particles.[18] A variable number of large lipid droplets stain red with Nile blue sulfate. These are found in increased numbers in degenerating cells in old tumors and usually in virus infected cells.[7, 8, 10] In addition finely granular or amorphous acidic periodic acid Schiff positive (PAS positive) lipid is found chiefly in the nuclear hof area of the cytoplasm (FIGURE 3b). In smears fixed in formol sublimate this material gives a positive acid hematin reaction for phospholipid;[19] it is also stained by the Kolatchev technique for demonstrating the Golgi apparatus[20] and is present in the region of the cell where the Golgi apparatus is demonstrable by electron microscopy (FIGURE 4). This phospholipid component will be called the Golgi lipid. The third lipid-containing structure in the cytoplasm is the mitochondrion. The mitochondria are more regular in size than the Golgi lipid particles and are distributed more evenly throughout the cytoplasm[21] (FIGURE 5). In the degenerating cells of old tumors

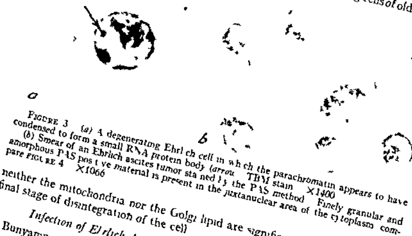


FIGURE 3. (a) A degenerating Ehrlich cell in which the parachromatin appears to have condensed to form a small RNA protein body (arrow). TBM stain. ×1400. (b) Smear of an Ehrlich ascites tumor stained by the PAS method. Finely granular and amorphous PAS positive material is present in the juxtanuclear area of the cytoplasm. Compare FIGURE 4. ×1066.

neither the mitochondria nor the Golgi lipid are significantly altered until the final stage of disintegration of the cell.

Infection of Ehrlich Ascites Tumor Cells with Bunyamwera Virus

Bunyamwera virus infection rapidly destroys the Ehrlich ascites tumor.[7] The earliest change detectable in the virus infected cell consists of margination of the chromatin and the parachromatin; the nucleus also is enlarged slightly and the chromatin forms a fine net enclosing the shrunken nucleoli. The nucleoli not only shrink but fragment so that as many as ten or more may be seen in a single cell (FIGURE 6a). The parachromatin appears to condense and possibly to increase to form one or several inclusions (FIGURE 6a and b). The inclusions are cytochemically indistinguishable from the RNA protein inclusions in degenerating cells of the uninfected tumor. Their acidophilia is blocked by deamination[22] and is therefore attributable to the presence of protein bound amino groups. The inclusion contains no DNA stainable by the Feulgen reaction but RNA can be demonstrated in them by the toluidine blue molybdate method.[7] The ultimate fate of the infected cells is depicted in FIG-

FIGURE 4 \n electron m crograph of an Ehrl ch ascites tumor cell show ng the aggrega t on of Golg meml anes and ves cles n the nuclear hof of the cytoplasm This photograph was prov ded through the courtesy of Darlene C Br ndley ×26 000



FIGURE 5 Smea of the Ehrl ch asc tes tumor sta ned by the tolu d ne blue m tochon d a sta n[20] ×1356

URE 7 The RNA protein inclusions are not extruded into the cytoplasm until the nucleus has disintegrated There is some increase in the neutral lipid droplets in the cytoplasm of cells showing nuclear changes but the Golgi lipid and the mitochondria are not altered detectably until the cell disintegrates



FIGURE 7 Final nuclear disintegration of Ehrlich ascites tumor cells infected with Bunyamwera virus modified Barrett stain ×1400

Infection of the Ehrlich Ascites Tumor Cell with Newcastle Disease Virus

Newcastle disease virus (NDV) has been adapted to infect and to destroy ... was thawed ac ... After one egg ... The first egg passage material was again inoculated into eggs and then into the Ehrlich ascites tumor *in vivo* by methods previously described[23] In each instance approximately 10 million tumor cells were inoculated intraperitoneally into Swiss mice followed 4 to 5 days later by inoculation of virus by the same route Smears of the peritoneal fluid were prepared daily stained by Leishman s

method, and examined for evidence of oncolysis.[23] The fluid was usually harvested and titrated[23] in eggs 72 hours after inoculation of virus. After 2 passages in the tumor, the virus was again inoculated into eggs. This egg-passaged material was then passed through the tumor *in vivo* for 6 passages, at the end of which 2 per cent of tumor cells in karyorrhexis were found in the smears, the LD_{50} titer of this fluid in eggs was 10^{-5}.[29] After 2 further passages in the tumor the LD_{50} titer of the fluid in eggs was greater than 10^{-5} and, in 3 different experiments, 16, 17, and 22 per cent of cells in karyorrhexis were noted in smears prepared 24 to 72 hours after infection (FIGURE 8). In smears of uninoculated control mice the incidence of karyorrhexis was never more than 1 per cent. Thus, after a total of 2 egg passages and 10 passages through the Ehrlich

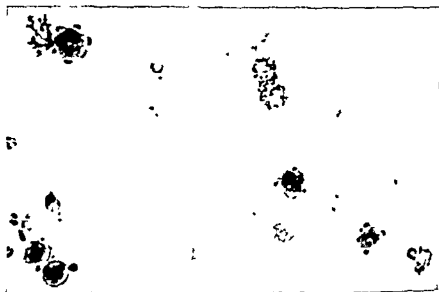


FIGURE 8. Smear of Ehrlich ascites tumor 72 hours after inoculation of the readapted NDV. Five cells in karyorrhexis and five cells with intact nuclei are present. Leishman stain. ×340

tumor *in vivo*, infection with NDV produced a marked cytopathogenic effect on the Ehrlich tumor.

The cytological changes in Ehrlich cells infected *in vivo* with the adapted NDV are similar to those described by Adams and Prince, who used the unadapted virus.[24] The first change detectable under the phase microscope 24 hours after inoculation of virus is the development of numerous inclusions in the Golgi zone of the cytoplasm (FIGURE 9*a* and *b*). The inclusions are less refractile than the bright neutral lipid globules of the uninfected tumor (FIGURE 10), they are often vacuolated and assume very irregular shapes (FIGURE 9*a* and *b*). Cytochemical tests reveal that the inclusions consist of doubly refractile PAS-positive acidic phospholipid (FIGURES 11 and 12). They are therefore, chemically similar to the Golgi lipid granules. On the other hand they are larger, often elongated and vacuolated and, in fixed material, they exhibit Maltese-cross birefringence.

In cells infected with NDV and stained by the toluidine blue molybdate method[17] the Golgi region which is the site of the phospholipid inclusions is clear or coarsely vacuolated (FIGURES 13*a* and *b*, 14*a* and *b*). The nucleus is

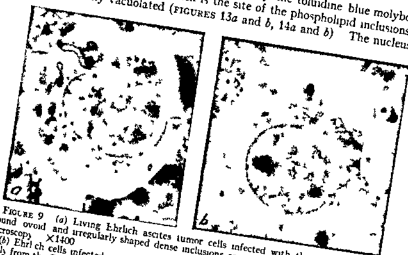


FIGURE 9. (*a*) Living Ehrlich ascites tumor cells infected with the readapted NDV. Round ovoid and irregularly shaped dense inclusions are present in the cytoplasm; phase microscopy. ×1400.
(*b*) Ehrlich cells infected with NDV. Elongated and club shaped inclusions project radially from the Golgi area of the cytoplasm; phase microscopy. ×1400.

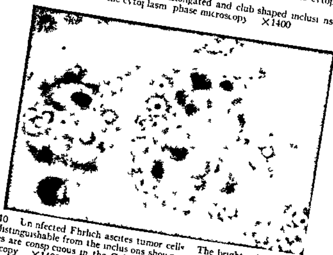


FIGURE 10. Uninfected Ehrlich ascites tumor cells. The bright refractile lipid droplets are readily distinguishable from the inclusions shown in FIGURES 9*b* and 10. Numerous minute vacuoles are conspicuous in the Golgi area between the two nuclei of the larger cell; phase microscopy. ×1400.

frequently indented and eccentric. The nucleoli are enlarged and the nucleolini are very prominent. The finely granular parachromatin frequently is concentrated in the indentation of the nucleus adjacent to the Golgi zone (FIGURES 13*a* and *b* and 14*b*). Minute buds of parachromatin often project from the nucleus in this region and as much as 35 per cent of infected tumor cells contain granules of RNA in the nuclear bof (FIGURES 13*b*, 14*a* and *b*). Only a small

proportion of these nuclear extrusions is associated with any DNA that can be detected by the Feulgen method The dinitrofluorobenzene and ninhydrin Schiff tests[25] indicate that protein is associated with the RNA Therefore



FIGURE 11 Inclusions in Ehrlich ascites cells infected with NDV PAS stain ×1600



FIGURE 12 (a) Uninfected and (b) NDV infected Ehrlich ascites tumor cells Note scanty large lipid droplets and finely granular Golgi lipid in the uninfected cells. Prominent round and ovoid lipid inclusions are stained in the Golgi zone of the infected cells Sudan black B ×1600

RNA protein, which is indistinguishable from the parachromatin is frequently extruded from the nucleus into the Golgi zone of the cytoplasm Comparison of the toluidine blue molybdate preparations with the phase microscope obser-

(FIGURE 9*b*) The presence of RNA protein of nuclear and possibly, of viral origin in the inclusions is consistent with the electron microscope observations of Adams and Prince.[24] The possibility that the inclusions may contain virus is suggested also by the demonstration of specific viral antigen in them.[26]

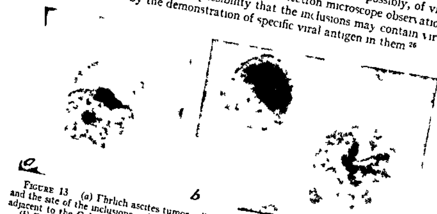


FIGURE 13 (*a*) Ehrlich ascites tumor cell infected with NDV. The nucleus is eccentric and the site of the inclusions is faintly vacuolated. Note the aggregation of parachromatin adjacent to the Golgi zone. TBM stain. ×1600.
(*b*) Two Ehrlich cells infected with NDV. RNA protein bodies are stained in the Golgi area and club-shaped processes of RNA protein protrude from the nucleus into the Golgi zone of one cell. TBM stain. ×1300.

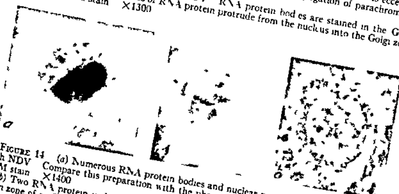


FIGURE 14 (*a*) Numerous RNA protein bodies and nuclear protrusions in a cell infected with NDV. Compare this preparation with the phase microscope photograph (FIGURE 9*b*). TBM stain. ×1400.
(*b*) Two RNA protein inclusions and a club shaped protrusion from the nucleus into the Golgi zone of an Ehrlich cell infected with NDV. Note the concentration of metachromatic (dark staining) parachromatin adjacent to the Golgi area. TBM stain. ×1130.
(*c*) Uninfected rat HeLa tumor cell. The round and ovoid mitochondria are evenly distributed throughout the cytoplasm. phase microscopy. ×960.

The development of the cytoplasmic inclusions is accompanied by an increase in the number and size of the neutral fat droplets of the cell. The mitochondria appear to be displaced by the accumulation of inclusions in the Golgi zone but they are not significantly altered until the final disintegration of the cell. Destruction of the cell is completed by nuclear shrinkage followed by karyorrhexis and disruption of the cytoplasm. Inclusions, nuclear fragments, and neutral fat droplets are liberated and frequently are seen inside the macrophages that replace the tumor cells in the ascitic fluid.

Infection of Rat HeLa Cells with Coxsackie B3 Virus

Inoculation of HeLa tissue cultures into the peritoneum of cortisone-treated irradiated rats produces large tumors.[11, 12] The growth of these tumors is not significantly affected by infection with Coxsackie B3 virus.[11, 12] After several



FIGURE 15. Smear of uninfected rat HeLa tumor. The Golgi lipid consists of flattened vesicles and minute granules. Sudan black B. ×1700.



FIGURE 16. (*a*) Prominent acidophilic inclusion in an uninfected rat HeLa cell; modified Barrett stain. ×1400.
(*b*) Circumscribed accumulation of fine granular material in the Golgi area of a rat HeLa cell infected with Coxsackie B3 virus. The nucleus (*arrow*) is shrunken; phase microscopy. ×1070.
(*c*) Smear of a rat HeLa cell infected with Coxsackie B3 virus. The pyknotic nucleus N is displaced by a circumscribed mass of sudanophilic material. Sudan black B and carmalum stain. ×2465.

passages of this virus through the rat HeLa tumor, a striking oncolytic effect is observed.[12] The cytological changes leading to destruction of the rat HeLa cell resemble those found in the Ehrlich cell infected with Newcastle virus. Before discussing the infected rat HeLa cell, a few aspects of the morphology and cytochemistry of the uninfected cell must be emphasized.

The appearance of the uninfected rat HeLa cell under the phase microscope is shown in FIGURE 14c. There are numerous round and ovoid mitochondria in the cytoplasm. The Golgi lipid gives a positive acid hematin reaction[19] and



FIGURE 17 Kolatchev Golgi stain of an uninfected rat HeLa tumor The Golgi apparatus is very minute in the healthy cells (*right*) and there is no significant increase in osmiophilic material in the degenerating cells (*left*) ×735

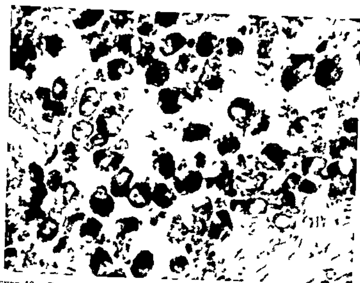
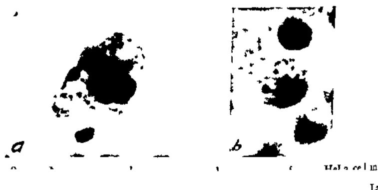


FIGURE 18 Conspicuous increase in osmiophilic material in rat HeLa cells infected with Coxsackie B3 virus Kolatchew Golgi preparation ×735

therefore contains phospholipid it stains with Sudan black (FIGURE 15) and by the PAS method FIGURE 16a shows an intranuclear RNA protein inclusion in an uninfected rat HeLa cell These inclusions are not uncommon in the nuclei of HeLa cells at the edge of areas of ischemic necrosis

Twenty four hours after inoculation of the adapted Coxsackie B3 virus, char

acteristic changes appear in the rat HeLa cells (FIGURE 16*b*). Under the phase microscope a circumscribed accumulation of granular material is seen in the Golgi zone of cells with pyknotic nuclei. The mitochondria are included in this material and the rest of the cytoplasm appears to be structureless (FIGURE 16*b*). In the more advanced stages of degeneration the mitochondria can no longer be recognized and the pyknotic nucleus is displaced or even indented by a dense vacuolated mass in the cytoplasm. The cytochemical and tinctorial properties of the cytoplasmic granular material are similar to those of the Golgi lipid" (FIGURES 16*c*, 17, 18). In addition to the lipid material, numerous very minute granules of ribonucleoprotein are often found in the Golgi zone" (FIGURE 19*a* and *b*). The nuclei of cells containing accumulations of Golgi lipid are shrunken and pyknotic and occasionally, minute spikelike projections into the Golgi zone are seen (FIGURE 19*b*). It is probable, therefore, that the RNA protein granules in the virus infected cells are derived from the nucleus in the same way as those found in Ehrlich cells infected with NDV.



HeLa cell in

La

Destruction of the nuclei of rat HeLa cells infected with Coxsackie virus is completed by karyolysis and fragmentation of the nucleus into dustlike particles. The cytoplasm persists *in situ* for several days and finally is removed by neutrophil granulocytes and macrophages.

Other Virus Infections of Tumor Cells Characterized by Increase in Golgi Type Lipid

Infection of the Ehrlich ascites tumor by Mengo, West Nile, and Anopheles A viruses results in oncolysis.[10] Each of these viruses produces an increase in the Golgi lipid similar to that observed with Coxsackie B3 and Newcastle disease virus infections. FIGURE 20 illustrates the great increase in this material following infection with West Nile virus.

In addition to changes in the Golgi lipid, well defined acidophilic structureless ovoid zones develop in the cytoplasm of practically every Ehrlich tumor cell ... from the Golgi

the virus induced necrotic regions described by Friedlaender *et al*[27] in electron micrographs of this material. They have not been observed in any other virus infected or uninfected cell.

Infection of the RPL-12 lymphoma of the chicken with St Louis encephalitis virus produces an increase in the Golgi lipid.[8] In smear preparations or frozen

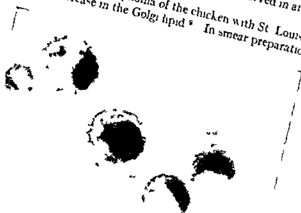


FIGURE 20. Striking increase in PAS positive lipid in the Golgi zone of Ehrlich ascites cells infected with West Nile virus (compare FIGURE 3b). PAS stain. ×1600.

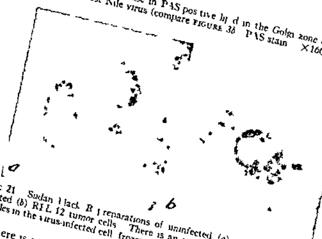


FIGURE 21. Sudan black B preparations of uninfected (a) and St Louis encephalitis virus infected (b) RPL 12 tumor cells. There is an increase in the number and size of the lipid particles in the virus-infected cell. frozen sections. ×2000.

sections there is an accumulation of PAS positive sudanophilic granules in the Golgi zone (FIGURE 21). This material persists in paraffin sections and fuses to simulate a faintly acidophilic inclusion body (FIGURE 22a). These inclusions are readily stained by the PAS method[8] as shown in FIGURE 22b.

Infection of the chicken lymphoma with St Louis encephalitis virus does not in itself lead to oncolysis.[8, 9] When this tumor is transplanted in the muscle the characteristic Golgi changes develop. Oncolysis is completed by phagocytosis of the abnormal cells. When the tumor is transplanted into the brain

the same changes develop in the Golgi lipid, but phagocytosis is deficient. The tumor cells continue to proliferate and kill the host.[6] Mitotic division of cells with hypertrophied Golgi lipid is however defective. Analysis of mitosis in the infected tumor reveals a significant increase in cells in metaphase, a decrease in those in telophase and an increase in binucleate forms (TABLE 1). This indicates some degree of metaphase arrest together with defective cytoplasmic division. The accumulation of lipid in the infected cells frequently displaces and even indents the nucleus. It is possible therefore that the pres-



TABLE 1
ANALYSIS OF MITOSIS AND BINUCLEATE CELLS IN RPL 12 LYMPHOMA INFECTED WITH ST LOUIS ENCEPHALITIS

Percentage of tumor cells in	Uninfected	Virus infected	Difference between means	Standard error of difference between means
Prophase	1.84	1.63	−0.21	0.19
Metaphase	2.51	4.88	+2.37	0.43
Anaphase	0.51	0.47	−0.04	0.13
Telophase	0.64	0.38	−0.26	0.13
Mitosis (total)	5.50	7.36	+1.86	1.44
Binucleate cells (per cent of tumor cells)	0.11	1.15	+1.04	0.19

ence of this lipid mass may interfere mechanically with the separation of chromosomes at the end of metaphase.

Discussion

Consi[illegible] infected tumors and in [illegible] certain [illegible] globules is a common feature of infected and uninfected degenerating cells. Alteration in the ribonucleoprotein metabolism of the nucleus in Bunyamwera virus infection is suggested by the frequent formation of numerous intranuclear RNA protein inclusions. Similar RNA protein inclusions are found much less

commonly in uninfected cells, in the rat HeLa cells, they appear to be the result of ischemia The great increase in the number of inclusions during virus infection may be due to disorganization of nuclear RNA metabolism associated with synthesis of virus In cells infected with Bunyamwera virus no alteration in the Golgi lipid can be found and the inclusions remain intranuclear until the nucleus disintegrates by karyorrhexis In Coxsackie B3 and NDV infection no intranuclear inclusions are formed, but there is a conspicuous alteration and increase in the Golgi lipid There is evidence that nuclear RNA-protein is extruded into the abnormal Golgi lipid where, at least in the case of NDV, it may be surrounded by phospholipid to form cytoplasmic inclusions RNA-protein of virus origin cannot be distinguished by present cytochemical methods from that of the cell In view of the demonstrable infectivity of the RNA from cells infected with a number of different viruses,[28, 29] it is possible that the RNA protein extruded from the nucleus may be concerned with the synthesis of the infectious particle Indeed, this type of developmental cycle has been postulated by Tournier *et al* [30] for varicella virus Simple particles are formed in the nucleus, from which they pass to the cytoplasm acquiring increasing morphologic complexity en route [30] A similar evolution of the infectious particle has been suggested by Fawcett in a study of the Lucke renal carcinoma of the frog [31]

An increase in the Golgi lipid, with or without the formation of cytoplasmic inclusions, has been found in six of seven virus infections studied This type of change has not been observed in uninfected tumor cells, but has been described in a number of other virus infections [9, 12] The development of juxtanuclear acidophil masses containing basophil granules has been reported in cells infected with poliomyelitis and Coxsackie B3 viruses [32, 35] Acidophil cytoplasmic inclusions also have been described by Brandt[36] in tissue cultures of chick embryo cells infected with NDV Although no histochemical studies have been made of these structures, the acidophil inclusions probably correspond to the Golgi phospholipid and the basophilic granules to the RNA protein particles observed in tumor cells infected with Coxsackie B3 and Newcastle disease viruses Electron microscope studies of monkey kidney cells infected with poliovirus reveal the development of a mass of U, or unknown bodies, in the juxtanuclear zone of the cytoplasm [37] Although Kallman *et al* [37] do not commit themselves as to the nature of the U bodies, they feel fairly confident that these bodies are not virus particles Since U bodies develop in the Golgi zone, they may be similar to the phospholipid accumulations found in this region in other virus infected cells

Although changes in the lipid in the Golgi zone are frequently found in virus-infected cells, there is little information to indicate any connection between these lipid changes and viral synthesis and replication The presence of virus like particles and of antigen in the inclusions of NDV has already been discussed Howatson and McCulloch[38] have described viruslike particles in a large osmiophilic inclusion in the Golgi zone of a mouse plasma cell tumor The particles resemble the viruslike bodies described in mammary tumors of mice [39] These authors have not yet been able to demonstrate that the particles are in fact viruses The teleological significance of the changes in the Golgi lipid in virus infection is not clear The hypertrophy and hyperplasia of the

Golgi lipid in cells of the RPL-12 lymphoma in the brain, which are infected with, but not destroyed L
paratus may have a defer
lipid bodies described by
cell and impede the development of the infectious particle

Summary

Infection of tumor cells with viruses produces changes comparable to those seen in nonneoplastic cells Certain virus infections produce cytochemically detectable disturbances in the RNA metabolism of the nucleus In the case of Bunyamwera virus infection, intranuclear ribonucleoprotein condensations or

eles A viruses, in chicken lymphoma cells infected with St Louis encephalitis virus, and in rat HeLa cells infected with Coxsackie B3 virus Many chicken lymphoma cells infected with St Louis encephalitis remain viable, but the abnormal accumulation of lipid in the Golgi zone appears to interfere with mitotic division Circumscribed structureless foci containing protein but no nucleic acid are found in the cytoplasm of Ehrlich ascites tumor cells infected with Anopheles A virus

Intranuclear RNA protein inclusions, like those in virus infected tumor cell have been found much less frequently in uninfected degenerating cells Although this abnormality of nuclear RNA protein can be produced by virus infection it may also result from ischemia and other causes

Acknowledgments

I express my gratitude to Hilary Koprowski, George R Sharpless and R Gerald Suskind without whose invaluable cooperation this work would not have been possible I also thank Julia A Douglas and Roscoe H Files for valuable technical assistance, and Gebhard Gsell for most of the photomicrography

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CELLULAR RESPONSES TO INFECTION WITH STRAINS OF HERPES SIMPLEX VIRUS

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Introduction

The characteristics of herpes infected tissues, both animal and egg have been well described In general, the tissues respond by ballooning of cells and the formation of multinucleated giant cells, the nuclei of the infected cells being altered by the formation of inclusion bodies Scherer[1] described the appearance of intranuclear inclusion bodies in strain L mouse fibroblasts, and later Scherer and Syverton[2] pointed out that infected HeLa cells became rounded and gathered in small clumps but in addition, they described a few foci of degeneration. Stulberg and Schapira[3] described the ability of herpes virus to grow on fibroblasts derived from various tissues of eleven-day-old chick embryos They reported the appearance of focal areas of necrosis with cells containing intranuclear inclusions at the margins of these areas

[illegible]

of syncytial giant cells and also by the piling up of rounded ballooned cells They utilized the technique of terminal dilutions to isolate different populations [illegible] cell respon e

[illegible]

apparent piling up They called the first virus population giant cell or GC strain, the second proliferative or P strain, and the third nonproliferative or NP strain

Development of Strains

Further observations have suggested that a true difference exists between the virus strain that produces giant cells and that which does not, and that the exact picture of the latter may depend on circumstances such as the char [illegible] the infection there [illegible] iono [illegible] edge [illegible] nped

cells of either type resemble bunches of grapes on the glass In this paper the strains will be designated as GC for syncytial giant cell producing virus and P for nongiant cell producing virus

More precise separation of the P and GC strains has been achieved by utilizing the technique of Kaplan [6] Herpes virus is grown on rabbit kidney tissue culture under agar and isolated plaques are picked for subculture By this means two strains GCA_3 and PA_5 have been isolated and studied and most of the responses demonstrated in this paper have been produced by these two subcultures TABLE 1 shows the source of these two strains and the labora

tory manipulations that they have undergone Both strains were derived from human clinical lesions

It should be noted in passing that when virus has been isolated from human lesions, whether primary or recurrent, and in tissue culture whether HeLa, human amnion, or rabbit kidney, careful observation of the original tubes has

TABLE 1
SOURCE OF STRAINS

GCA_4

From recurrent vesicle, May 23, 1955
- ↓ CAM
  - ↓ HeLa = mixed May 25 1955
  - ↓ Frozen at −70° C
  - ↓ HeLa April 29 1957
  - ↓ HeLa (45)
  - ↓ Line amnion under immune serum Jan 20, 1958
  - ↓ Plaque
  - ↓ Line amnion (5)
  - ↓ Rabbit kidney, Feb 20 1958
  - ↓ Rabbit kidney under agar, (10) May 29 1958
  - ↓ Plaque = GCA_4
  - ↓ Rabbit kidney (6)
  - ↓ Stock virus at −70° C
- ↓ Frozen
  - ↓ HeLa = chiefly GC

(Frozen at −70° C through Line amnion under immune serum: kept at −70° C at intervals)

PA_5

From vulval vesicle March 7 1957
↓ HeLa = chiefly P
↓ HeLa (19) kept at −70° C in intervals
↓ Rabbit kidney under agar May 27 1958
↓ Plaque = PA_5
↓ Rabbit kidney (4
↓ Stock virus at −70°

Numbers in parentheses indicate number of passages

usually revealed evidence of both types of response In our experience it is more usual to find primarily a proliferative response with few if any giant cells Occasionally giant cells seem to predominate

Giant Cell Strain (GC)

The histological changes seen in the giant cell strain are as follows In an ill-defined focus of infection among the normal cells may be seen a group of

scattered nuclei, which on close inspection seem to be those of swollen cells, the boundaries of which are contiguous or absent as the intervening membranes apparently lyse Normal-appearing cells may be seen at the margin as if about to be incorporated Later, the area of fusion enlarges and the nuclei appear to ... together Still later there appears to be shrinkage which ... massed in the middle ... fication it may be seen that most of the nuclei ... n bodies, although



FIGURE 1 Early formation of syncytial giant cell The nuclei are seen lying separated by cytoplasm the boundaries of the cells sometimes may be distinguished Rabbit kidney, Giemsa stain tube ×75

some could not be distinguished readily from normal cells (FIGURES 1 to 5) Occasionally individual nuclei are very large, and sometimes, in a cell, one or several nuclei, each containing an inclusion body, appear as if they may have just divided

P Strain

... appear on top of the monolayer ... piled up area of single rounded cells protrudes ... passes, the surrounding cells of the monolayer seem to become attached to ... pile, so that the focus lies as if shrunken away from the monolayer and finally only piles of cells, like bunches of grapes may be seen attached to the glass The rounded cells, form-



FIGURE 2 More ad anced les on th several masses of nucle ly ng n empt a eas in wh h no cell boundar es may l e seen Rabb t k dney G emsa stain tube ×68

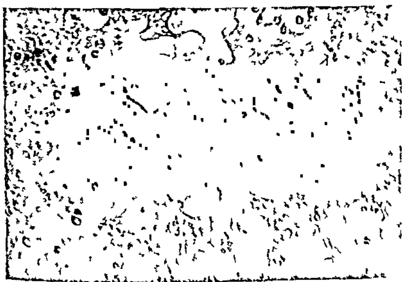


FIGURE 3 Syncytial format on sim lar to FIGURE 2 but in HeLa cel s L ving culture HeLa tube ×68

FIGURE 4 Late appearance of giant cells in which they are rounded and apparently contracted with central masses of nuclei surrounded by cytoplasm Living culture rabbit kidney tube ×68

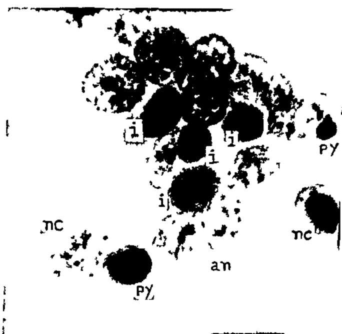


FIGURE 5 Intermediate stage syncytial giant cell showing appearance of intranuclear inclusions *i* of various stages, as well as some nuclei that are altered little *an* Two dead cells with pyknotic nuclei *py* are present and two normal cells *nc* in contact with the edge of the giant cell HeLa H and E stain tube ×300

ing the pocklike focus, chiefly show evidence of infection by the presence of inclusion bodies. Some of the cells at the edge may have normal appearing nuclei but in a field of such thickness it is difficult to distinguish nuclear details (FIGURES 6 to 10).

The host cells that respond with proliferation not infrequently also produce a small number of small giant cells, but the syncytial masses are never seen. This striking difference can be demonstrated in comparing very advanced states of each strain on primary human amnion cells (FIGURES 11 and 12).

Effect on Pure Fibroblasts

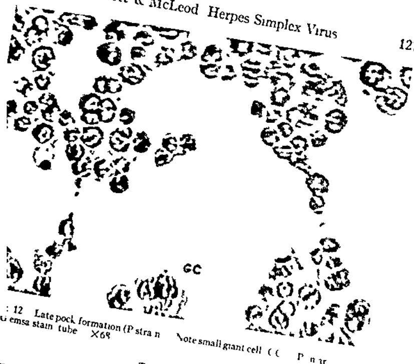
In addition to producing the effect of these two strains on HeLa, amnion and rabbit kidney tissue it was also possible to infect chick embryo fibroblasts derived from eleven-day-old chick embryos with both strains. In this tissue it was easy to find giant cells produced by the GC strain. The pocks of the P strain were harder to recognize because of the irregular nature of the monolayer, but we never saw syncytial formation with the P strain and thought that we could recognize proliferative foci in the stained if not in the living tissue.

Other Characteristics

It was obviously of interest to determine if these two populations of virus particles had other differentiating characteristics that might suggest genetic markers. The information available is summarized in TABLE 2 and is very disappointing from that point of view. Titration in tubes indicates that both strains grow almost equally in the various hosts tested. Although the data presented show a tendency for the GCA_3 strain to have a higher titer than the PA_8 strain, this difference is not consistent. The GCA_3 plaques formed on the rabbit kidney cells do appear to be somewhat larger (4 to 5 mm in diameter) than the PA_8 plaques, 2 to 3 mm in diameter; however there is sufficient variation in each to make consistent recognition difficult. On more recent studies with controlled pH, there appears to be no difference in the rate of heat degradation at 37° C of the two strains, although we had thought from earlier experiments that the GC strain was less sensitive. Ultraviolet light appears to inactivate both strains at the same rate. Both strains adsorb onto rabbit kidney cells at the same rate, 75 and 80 per cent of the GCA_3 and PA_8, respectively, being adsorbed in 2 hours whereas newly formed virus in each instance appears in the medium after 7 hours. Electron microscope pictures thus far have not revealed any consistent difference between the sizes of the virus particles derived from the two strains. Each strain has retained its pathogenicity for animals; there is no difference in titer in weanling mice and rabbits respond to corneal scarification by the development of a keratoconjunctivitis without encephalitis. The response of the chorioallantoic membrane of the embryonated hen's egg (CAM) deserves some comment. It is interesting that both strains produce typical pocks, again there is a suggestion that the P pocks are smaller than the GC pocks, but the difference is obviously insufficient to be a useful marker. Passage of each strain in CAM for as many as eight passages did not appear to alter its capacity to produce a specific response in tissue culture: the passaged P strain produced piled up pocks and the passaged GC strain syncytial giant cells.

FIGURE 10. Edge of pock showing intranuclear inclusion bodies in most of the cells in focus at the top of the pile. HeLa, H and E stain, tube. ×270

FIGURE 11. Late giant cell formation (GC strain). Primary human amnion, Giemsa stain, tube. ×68



: 12 Late pock formation (P stra n ... Note small giant cell ((... P n ar
...on G emsa stain tube ×68

TABLE 2
COMPARISON OF STRAINS

Hosts	GCA	PA
RK cells ID₅₀/ml	$10^{-[illegible]}$	$10^{[illegible]}$
RK cells PFU/ml	1.4×10^{8} larger?	1.5×10^{6} smaller?
HeLa cells ID_{50}/ml	$10^{-[illegible]}$	$10^{[illegible]}$
Chick embryo fibroblast cells ID_{50}/ml	$10^{-[illegible]}$	$10^{[illegible]}$
CAM PFU/ml	2×10^{6} larger?)	2×10^{6} smaller?
Weanling mice LD_{50}/ml	$10^{[illegible]}$	$10^{[illegible]}$
Rabbits cornea keratoconjunctivitis	+	+
encephalitis	−	
Heat degradation at 37° C ½ life	2.2–3.3 hours	2.0–3.5 hours
Ultraviolet inactivation at 8 in distance	9–12 min	9–12 min

Summary

In summary it would appear that it is possible to derive from freshly isolated human herpes virus at least two types of infecting agent one of which leads to the breakdown of the boundaries of the host cells in the process of infection with the formation of syncytial giant cells the other appears to produce a piling up and apparent proliferation of the host cells without any tendency for cell boundaries to lyse Since these responses can be produced at will it may

TABLE 1

MULTIPLICATION OF ISOLATED HELA CELLS IN MICRODROPS FOLLOWING EXPOSURE TO HERPES VIRUS

Multiplicity of infection HeLa PFU per cell	24 hours		72 hours		Observed proportion of cells with inhibition of mitosis*	Theoretical proportion infected with one or more infectious units
	Total examined	Proportion divided	Total examined	Proportion divided		
uninfected	181	0 46	181	0 76	—	—
0 1	90	0 28	70	0 68	0 10	0 09
1 0	120	0 12	40	0 27	0 64	0 63
2 7	40	0 05	40	0 12	0 84	0 93

* Proportion of cells in infected cultures dividing by 72 hours / Proportion of cells in uninfected cultures dividing by 72 hours

Virus treated at 50° C for 1 hour had no antimitotic activity, and the antimitotic activity was reduced by addition of herpes antiserum Additional normal serum also had a slight protective effect on the cells, but the difference was significantly less than that produced by antiserum

Assuming that 76 per cent of the cells would have divided by 72 hours if they had not been exposed to virus, it is possible to calculate the proportion in which division was inhibited This is found to agree closely with the theoretical number of cells adsorbing one or more HeLa PFU as deduced from the Poisson distribution We can conclude that one HeLa PFU is sufficient to inhibit mitosis, because about half the cells affected should not have absorbed more than one PFU per cell

It is known from other experiments that there are about ten times as many chick cell infective particles as HeLa infective particles in the seed suspension, and it is also known that the chick cell infective particles adsorb to the HeLa cells, although the majority do not initiate infection (Stoker and Ross, 1958) In the experiments with single HeLa cells described above, a multiplicity of 0 1 HeLa PFU per cell would probably be accompanied by adsorption of about 1 0 chick cell infective unit per cell It is obvious from the high proportion of dividing cells that this does not inhibit mitosis

With the higher doses of virus the proportion of cells releasing infective virus into the microdrops was small and not related to input This failure of virus release from cells which nevertheless initiate plaques (infective centers) in uninfected HeLa cells is discussed elsewhere (Stoker, 1959), but it should be noted that none of the cells yielding virus divided, none formed multinucleate giants the majority were rounded, and some had already disappeared when the sample containing virus was taken

Kinetics of Mitotic Inhibition

... to inhibit mitosis, we investigated the ... akes place This was ... (parasynchronously),

When exponentially growing monolayers of HeLa cells at 37° C are chilled to 4° C for one hour, then returned to 37° C, the cell numbers remain almost

stationary for 18 hours The majority of cells then divide within 1 to 2 hours This is followed by a stationary phase of 18 hours and then a second increase in cell numbers lasting 1 to 2 hours Thereafter the cultures become too crowded for exponential division The proportion of normal cells dividing usually varies from 60 to 80 per cent Those cells that do not divide para synchronously never divide, and so they do not confuse the steplike pattern of growth Full details of the procedure are given by Newton and Wildy (1959)

To investigate the time between virus attachment and inhibition of mitosis, replicate cultures of HeLa cells were chilled and at different time intervals during the first stationary phase were exposed to herpes virus suspension for an adsorption period of one hour, after which herpes antiserum was added to prevent further infection The dose of virus was sufficient to infect about two thirds of the cells Parallel cultures were inoculated with medium containing no virus Cell counts were made at intervals on sets of four or more replicate cultures

The uninfected cultures and cultures exposed to virus and antiserum divided at 18 hours and again at about 36 hours, while the majority of cells in the infected cultures failed to divide at all The inhibition of cell division took place even when the virus was first added 16½ hours after chilling 90 min before expected division Since neither infection nor cell division are quite synchronous, it is not possible to estimate accurately from this the time interval that elapses between virus adsorption and the damage to mitosis Adsorption is rather slow, however, and 1 hour must elapse before sufficient virus attaches to infect two thirds of the cells This would have been complete 17½ hours after chilling, or 30 min before cell division should have commenced It seems probable that the damage to mitosis occurs within 60 min of virus attachment and, of course, it may be much less This may be compared to a lag phase of 9 to 12 hours before new virus appears in the cell fraction, and it suggests that as *in certain types of bacteriophage, damage to mitosis is a relatively early event* in the cycle of virus replication It is of some interest that this inter

of them retain certain of the characteristics of living cells They remain rounded, although morphologically intact They stain with neutral red and not with trypan blue Production of acid suggests that in some respects they are more active metabolically than uninfected cells In intact monolayer cul-

this need not apply to other strains of herpes virus or other cells For example,

lactory evidence for this process has been found only with Rous sarcoma virus in cultured chick cells (H Temin and H Rubin, unpublished data)

The tissue changes induced by several viruses seem to suggest proliferation of infected cells, but it is not possible to define accurately the relationship between virus growth and cell division without quantitative studies *in vitro*. Although our investigations showed no evidence of continued division of the infected cell, the techniques involved may be applicable to the investigation of other virus-cell systems.

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ELECTRON MICROSCOPE STUDIES OF CELLS INFECTED WITH THE SALIVARY GLAND VIRUSES*

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Electron microscopy has stimulated morphologic study of viral diseases in articular, since visualization of the infective agent is now possible Particles ssociated with many virus diseases have already been identified and work has egun in the complex area of viral life cycles The mechanisms whereby vi uses gain access to the interior of cells, the fate of virus particles after pene ation of the cell, and the site and type of morphologic changes accompanying ral growth all need to be examined With these problems in mind we shall resent here not only the alterations occurring in distinctly diseased cells, but so early changes evident after the addition of human salivary gland virus to ssue cultures of human fibroblasts

Materials and Methods

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ere maintained in a medium composed of 85 per cent beef amniotic fluid, 10 er cent beef embryo extract, and 5 per cent horse serum All media contained 00 U of penicillin and 100 μg of streptomycin per ml Four series of experi ents were carried out In 2 of these, pooled media from cultures infected 10 ays previously were filtered through a 015 Celas filter, and an inoculum of 0 5 l of the filtrate was added to 1 5 ml of fresh media for each roller tube In ne other 2 series, media from infected cultures were centrifuged and 0 5 ml f supernatant added to the fresh media of each tube Cultures were fixed at tervals following inoculation Parallel control cultures were obtained at 2 o 3-day intervals Immediate control cultures received an inoculum of super atant from uninfected cultures equal in amount to the infected supernatant dded to experimental cultures

Both normal and infected cells were prepared for electron microscopy by xation in Dalton's osmic acid fixative (1 per cent osmium tetroxide in bichro- nate) for 10 to 15 min [2] Cells were fixed and dehydrated *in situ* in the roller ube after decanting the culture media Dehydration was by graded solutions f ethanol (10 to 100 per cent) over a total period of 1 hour The cell colonies ere removed from the tube with a dural elevator and placed in one half ab- olute ethanol and one half methacrylate They were infiltrated in 3 changes f 7 1 butyl methyl methacrylate for a total of 1 hour A catalyst benzoyl eroxide, was added to the last change of the plastic The fibroblasts were mbedded in gelatin capsules filled with the slightly polymerized methacrylate mixture Polymerization was at 60° C for 12 hours Blocks were sectioned

* Th ... B 1539 Na

Original electronmicrographs were made at 2000 to 12,000 diameters on Cramer contrast plates, developed in Kodak D-61 diluted one half with water and subsequently enlarged photographically as desired.

Results

Control. Normal fibroblasts in tissue culture were elongated cells with rather scant cytoplasm that often extended into attenuated prolongations (FIGURE 1). The nuclei were ovoid, although occasionally indented with one or more prominent nucleoli. The nuclear chromatin was finely granular and



FIGURE 1. Electron micrograph of normal elongated fibroblast in tissue culture. Part of the nucleus is evident at the left. The cytoplasm contains elongated narrow ergastoplasmic sacs. Mitochondria are scant and small. Approximately ×6000.

uniformly distributed with a distinct increase in density near the nuclear membrane. Mitotic figures were rare. The cytoplasmic ergastoplasm was arranged as parallel rows of membranous envelopes with more or less prominent dense granules present along their outer margins. The inconspicuous Golgi material was evident as clusters of small vesicles in the perinuclear region. Mitochondria were sparse and elongated with distinct cristae. Occasional intracytoplasmic inclusions of lipid occurred. A zone of fine intracytoplasmic tonofibrils was present at the inner margin of the plasma membrane of some fibroblasts. Normal fibroblasts occasionally had moderately abundant cytoplasm rather than being spindle shaped. Cells of control cultures to which supernate from noninfected cultures had been added a few minutes prior to fixation showed some dilatation of ergastoplasmic sacs and a questionable increase in cytoplasmic vesicles in comparison to cells of cultures of the same age incubated for 2 or 3 days without change of the media. A majority of the cells remained

spindle shaped and the changes were minimal in comparison with those of cells [illegible]

[illegible] s after [illegible] ular in both the cultures examined immediately and in those examined 5 min after inoculation, consisted of a rounding up of cells with an apparent increase in



FIGURE 2 Electron [illegible] virus [illegible] is plump [illegible] toplasm [illegible]

cytoplasmic volume There were numerous pinocytosis vacuoles evident (FIGURE 2), some of these contained electron dense material The ergastoplasmic sacs were frequently dilated and filled with an opaque homogeneous material There was a multitude of minute vesicles within the cytoplasm (FIGURES 2 and 3) Mitochondria were unaltered For the most part the nuclei were unaltered, but an irregular rim of dense material was present at the nuclear margin in some cells

Viral particles were not identified at the outer margins of cells or between

abnormal although the nuclei remained relatively unchanged. The cytoplasm was increased in amount and many cells were almost spherical (FIGURE 8). Small mitochondria were numerous. The ergastoplasmic sacs of some cells



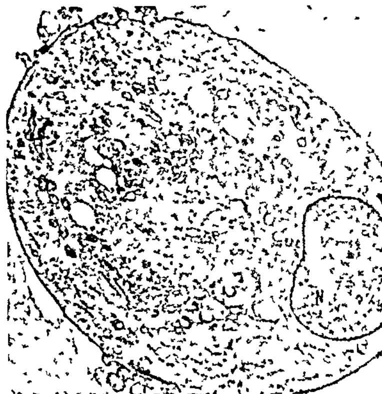
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were distended, in others they were inconspicuous, the cytoplasm being filled with numerous small vesicles. The Golgi substance was prominent.

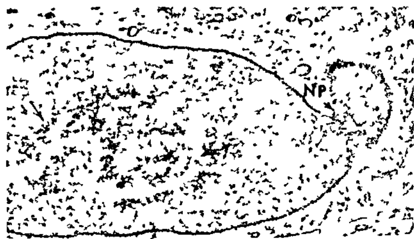
The earliest definitely abnormal nuclei with nuclear inclusions were identified 2 to 3 days after inoculation. At this time the central portion of the nucleus was partially filled with an inclusion containing particles composed of a small dense central body surrounded by a pale halo and an outer dense membrane

(FIGURE 9) The particles in these inclusions measured approximately 60 to 90 mμ in diameter[4] They were interspersed within a more dense granular material and the entire inclusion was surrounded by a pale region between it and the nuclear membrane that was distinctly increased in density During development of a nuclear inclusion the number and density of particles increased (FIGURE 10) Nucleoli remained unaltered or became somewhat more



prominent No stage of altered nuclear morphology preceding the appearance of viral particles was identified definitely At the time of the appearance of particles and of the nuclear inclusion identifiable virus particles were not evident in the cytoplasm

pl e
ou c
viral particles have been described previously[4] Two types of cytoplasmic



fection



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particles associated with infected cells were recognized (FIGURE 13). One averaging about 80 to 100 mμ in diameter was a target form comprised of a central dense dot surrounded by a single or double rim of dense homogeneous material. The other type of particle consisted of a solid sphere varying from 150 to over 500 mμ in diameter. Both types of particles were within membranes some of

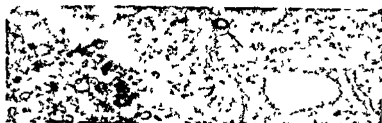


FIGURE 11 The double nuclear membrane is separated at the upper right (arrow) and encloses a dense particle that is similar to the mature type of particle seen in the cytoplasm (compare with FIGURE 13) ×25 000

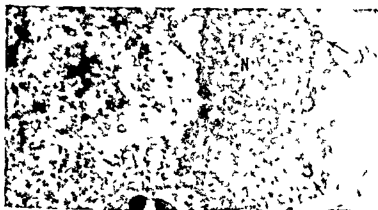


FIGURE 12 A section through part of the nucleus of another fibroblast infected 10 days previously The nucleolus (N) apparently is unaltered At the arrows 2 particles similar to the one seen in FIGURE 11 are present between the two membranes surrounding the nucleus ×20 000

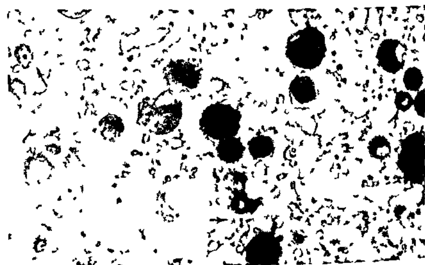
which appeared to be those of the ergastoplasm In the older cultures numerous free particles were noted between cells

Discussion

penetration of the cell since the latter occurs with such rapidity From these studies it appears that the process by which virus particles gain access to the cell is not one of direct combination of the particle with the cell surface but rather one of engulfment Indeed rather large masses of material are evident within the cytoplasm of some fibroblasts (FIGURE 7) Following addition of media from cultures of uninfected fibroblasts control cultures show only a minimal increase in pinocytosis or dilatation of ergastoplasmic sacs Rapid and

extensive pinocytosis may be due to addition of protein which stimulates the process.[5] We have not yet examined cultures following addition of heat killed virus, and do not know whether addition of killed viral suspensions would lead to a response similar to that containing viable virus. The rapidity of pinocytosis frequently observed in living tissue culture cells is neither surprising nor unusual.[6]

The fate of virus particles during and after entrance into the cell remains unknown. Occasionally particles reminiscent of target forms of the virus are seen within cells recently exposed to virus. Whether these forms are identical with those in the inoculum is not known. The definitive changes in virus particles during penetration of the host cell are not yet understood. The virus



may well disappear secondary to a structural alteration of protein molecules after or during penetration of the cell membrane.

The late alterations of cells infected by salivary gland virus have been described by both light[7, 9] and electron[4] microscopy. The early changes prior to the classic cytomegalic picture, however, have not been described in any detail previously. As we have shown, the fibroblast infected 10 to 20 days previously is a large cell containing both nuclear and cytoplasmic viral particles. In light of this, it is most interesting to see early cytoplasmic changes with ingestion of particulate material and enlargement and rounding up of the cell but with no early nuclear alteration. Two to 3 days later, a nuclear inclusion of viral particles has formed, while none is evident in the cytoplasm until about the fifth day after infection. This raises the question of the manner in which infective material reaches the nucleus, as well as the question of our ability to recognize early pathological alteration of chromatin.

The nucleus certainly is not isolated from the cytopla n Wat ⁿ and others have clearly shown that the ergastoplasmic sac are nt u th the outer nuclear membrane as well as with the plasma memb ane We h ve seen mature virus particles between the two nuclear membrane n in fected cells[2] (FIGURES 12 and 13) That a virus may lea e the nu leu d reach the cytoplasm is however, a different matter th n pe ri t

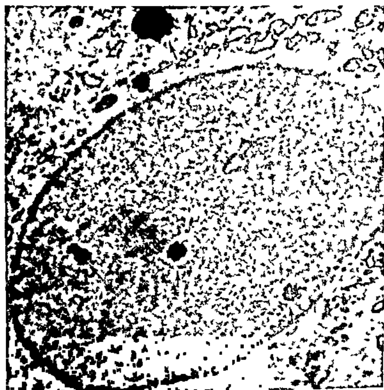
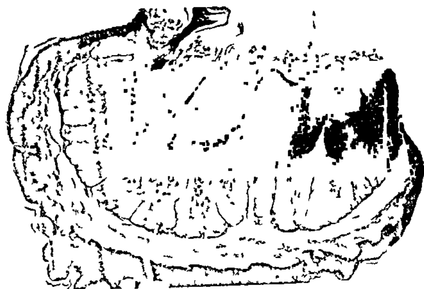


FIGURE 14 Electron micrograph of the nucleus and part of the cytoplasm of a rabbit yolk sac epithel al cell T enty four hours previously India ink ha been njected nto the u er ne lumen Ink (*arrows*) is present in both the nucleus and cytoplasm ×12 000

intact nucleus by extracellular or intracytoplasmic foreign particles There is definite evidence that the latter does occur Following injection of a suspen s on of colloidal carbon (India ink) into the uterine lumen of the pregnant rab bit yo [illegible] aggregates of carb [illegible] *et al*[14] [illegible] in nuc [illegible] eign protein Whether these materials entered the nucleus by means of the well-demonstrated nuclear pores[10] or by a mechani m akin to pinocytosi [15] by the nuclear membrane is not clear at this time

basal layer with the inclusion bodies becoming increasingly evident and maturing toward the surface

The nucleus is pushed to one side and the nuclear membrane is markedly folded The nucleus contains comparatively large dense granules sparsely scattered throughout the nucleoplasm which distinguishes it from the nucleus of the uninfected cell Mitochondria with their characteristic cristae are seen at the periphery of the cell Near the nucleus are several other bodies that do not have cristae but seem to be associated with the viral infection They are sur



F S n of moll scum contagiosum surrounded by epiderm s wh ch appears are enlarged Inclus ons appear in the s covered by a mass of molluscum bod e

rounded by a membrane and have a granular content There is a thin rim of cytoplasm separating nucleus and inclusion body from the cell membrane and within the cytoplasm a few strands of keratin are present The inclusion body contains locules of elementary bodies separated by septa of complex composition in which varying forms of the developing elementary bodies are present The entire cell is surrounded by a convoluted membrane This is the general picture of an infected cell How this picture evolved how it progresses and the finer details of the various structures mentioned are of considerable interest

Nucleus In an infected cell the nucleus and nucleolus are usually enlarged but as the inclusion grows the nucleus apparently shrinks and large folds appear in the nuclear membrane (FIGURE 3) The nucleus is pushed to one side becomes flattened and on sectioning appears elongated Unique to the nucleus of the infected cell are relatively large dark bodies of varying size and of slightly

ir egular shape that are present n ar able number and a l gh the nu leoplasm

Cy opla m The organelles of the infected cell an l on ke n a e pu hed as de by the develop ng n lus on al ho om en



F RE 2 Ce l gr a y d end d by a mo uscum o y Th nu u n h u e pa o he p u e Th e s a hun m o o la m ad a n o h ce wa con a n ng e organ es No e he la g d nse granu s n he nu us an h C bo × 300

rapped Tl e m tocl on lr a are d pla ed and oc a na ly are lump ed (F G URES 4 and 5)

Bod es of the o der of 22 to 950 mμ are of en een n the y topla m about tl e nu leu or even at a d tance from t and are des gna ed a C for cy opla m

bodies (FIGURES 6 and 7) The larger ones often have a distinctly double membrane measuring ≈ 165 Å in thickness with the space between the two unit [illegible]

stances the membrane appears to have overlapped itself since 3 dense unit membranes are seen The contents of these structures are usually sparse and composed of reticular and granular material, although relatively large bodies or an occasional vesicle may be present within them The double membrane



[illegible] leoplasm

surrounding them appears coarser than the membranes of the mitochondria present in the same photograph, and the unit membranes themselves are wider and darker than most of the mitochondrial unit membranes When contrasted with the nuclear membrane, it is darker and does not have a surrounding condensed rim of cytoplasm

Developmental structure of inclusion body In the earlier stages of infection there appears in the cytoplasm of the cell material similar to that making up the septa of the more mature inclusion body This early inclusion pushes back the mitochondria and the nucleus (FIGURES 2 and 4) In this respect it seems to replace the cytoplasm, but it does not do so entirely, for at least one component, the keratin fibrils, is often entrapped (FIGURES 5 and 8)

The mater al compos ng the early nclus on conta n t vo types of ground stru tures one s finely ret cular although t may appear amorphous at lo v magn ficat ons and the otl er granular occurr ng n lo alized areas (FIGURES 2 9 and 10) The ret ular fibr l are very fine some at the l m of resolut on of the m roscope of en v th t ny den e nodes at the r po n of n er ect on (F URE 10) The granules of the granular component are n the order of 130 o 160 Å n d ameter and are apparen ly embedded n a e ond sub an e (F GURE 9) That to say t ny strands can be seen runn ng be een the



g anule or they may be nd st nct because of an amorphous mater al surround ng them

W h n the ground sub tance of the early nclu on other structu es are p es en (F GURE 11) One s a round or oval profile rang ng from 640 to over 3000 Å They are surrounded by a membrane h the nter or clear to moderately den e The contents are e ther homogeneous conta n a *dense bar or there s* a suggest on of a fe granules The second type of profile s that of a dense granule n the order of 250 to 850 Å n d ameter surrounded by the maller g anu es of the granular ground substance All these structures generally occur n foc and are asso ated w th the granular ground substan e

The above structures are clearly part of the early nclus on There are

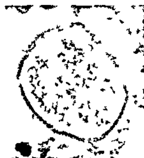
FIGURE 5 [illegible]



FIGURE 6 *C* bodies (*C* and *brackets*) in cytoplasm Portion of nucleus at bottom ×7400

others however that probably appear later and are seen regularly in the septa of older molluscum bodies These are of four types and do not eem to be

a crudely elliptical profile in the order of 90 mμ in diameter it i separated



F GURE 7 C body Note granular an l ret cular nner structure doul le men l rane nd the fibr llar nature of the tangent ally cut membrane ×36 000



FIGURE
sul tance
per cent
osm um t

from the rest of the contents by a less den e zone (FIGURE 12b) The nucleoid is often much den er than the rest of the contents wh ch are also reticular The s ze of the nucleoid varies although some of the var at on in size and den s ty may be the result of tangential sectioning It is often pre ent in a larger structure than Type II and at times the nucle l-contain ng den e central port on has condensed leaving a clear zone between it and the surrounding membrane (FIGURE 13)

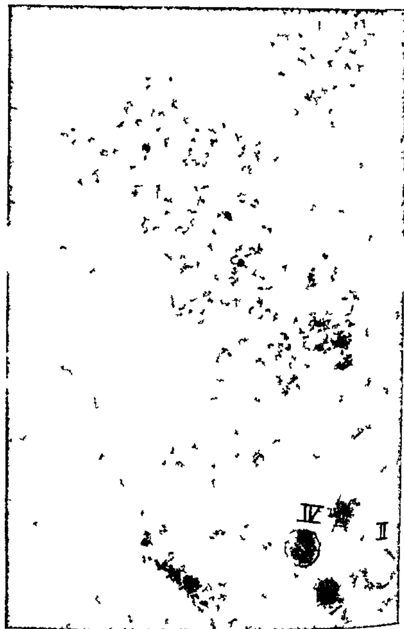


FIGURE 9 Re cula ground subs ance and focal a eas of granular ground sub ance of the molluscum body Type II and Type IV de elop men al fo ms of the e ementary body are p esent n the lo ght S o en fo ms of the ma ure elemen a y body a e a so p esen ×44 000

Type II is a round compact body with or without a membrane (FIGURE 14) The membrane which
and appears single
making up the body
rat ng it from the membrane In such a structure a den er eccentric area

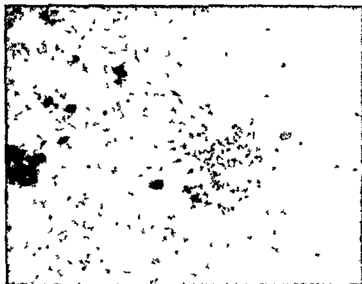


FIGURE 10 Reticular and granular ground substance Note nodal th ckenings at po nts of intersect on of fine filaments of the ret cular ground subs ance Granular groun l substan e s clumped Large dark bod es are port ons of elementary l odies A fe kera in f l r ls are present fixation and staining as in FIGURE 8 ×17 000

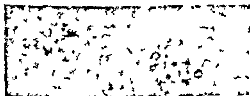


FIGURE 11 Reticular ground sul stance surroun l ng foci of g anular g oun l sul tance These foci conta n round and oval profiles and relati ely large dense granules w th sn all granules clustered about them ×19 000

suggest ve of a disappearing nucleo d sometimes i seen in the central den e plasma (FIGURE 15)

Type III is a lobulated structure of a reticular moderately den e material with
Ty
dense
to be seen least frequently

Elementary bodies The forms of the virus that occur predominantly within the locules are considered the mature elementary bodies They are of two types, one distended and the other condensed, together with intermediates (FIGURES 2 and 18) As a general rule, the elementary bodies adjacent to the septa or in the inclusions of the lower Malpighian layer tend to be distended, whereas those in the inclusions far removed from the basal layer are predominantly, if not exclusively, condensed Between the elementary bodies is a

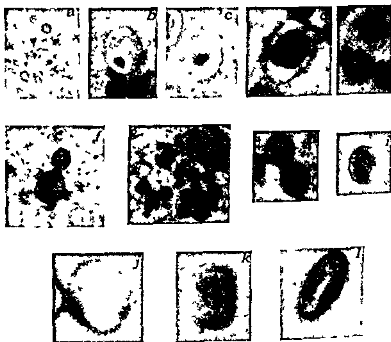


FIGURE 12 Postulated sequence of developmental forms resulting in mature elementary body (a) Large dense granules surrounded by zones of smaller granules of the granu ... ×10 000 (b) Type I nucleoid-containing body ... g membrane ×43 000 (d) Pos... 000 (e) Type II ×31 000 (f) ... I lobulated form ×16 000 (h) ... form of mature elementary body ... ody sectioned through rectangular ... ary body sectioned almost normal

very sparse and fine reticulum that extends from their surfaces (FIGURES 19 and 20)

The structure of the condensed form of an elementary body is seen in FIGURES 20 and 21 It is a rectangular body that appears as an ellipse in sections normal to the rectangular plane There is an outer membrane made up of two unit membranes and an inner membrane again composed of two unit membranes When fully collapsed the inner membrane takes the form of a biconcave disk When distended it surrounds a less dense central area of varying size depending upon the extent of distention The inner and outer

membranes are separated by homogeneous moderately dense wall material that increases in thickness as the central areas beneath the broad convex surfaces of the elementary body are approached In the rectangular plane



FIGURE 13 Type I nucleoid containing structure (*arrow*) Other developmental forms are present in the septa and mature elementary bodies in the locules ×21 000



FIGURE 14 Type II round to oval profiles with dense interiors Swollen forms of the mature elementary body are present ×20 000

however the two membranes often appear to meet at the margins FIGURE 22 is a drawing of the proposed basic structure of the elementary body

Within the mature elementary body is a dense granule (FIGURES 18 to 21) It i … from 80 to 100 Å in widt … It is often surr … hape Most

frequently it occurs associated with the thickened portion of the wall but may be seen on the inner membrane or within the less dense central area

Its occurrence in the mature elementary body is such that when the thick ness of the section is con idered together with the dimensions of the elementary body it would appear that there is one den e granule for each elementary body



FIGUR
Irane I
mentary

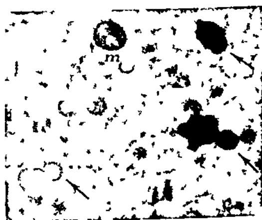


FIGURE 16 Type III germinating forms (*arrows*) M mitochondrium ×19 000

This dense granule also occurs in the d stended forms of the virus usually associated with the thickened portion of the wall however they may be seen occasionally within the less dense central area It has been seen in Types II and IV of the developing elementary body It has not yet been seen in Types I or III and does not seem to appear outside the elementary body Occasionally two bodies both of which could be interpreted as the dense granule are seen in the mature condensed elementary body or in the developmental forms



FIGURE 17 Type III, in cytoplasm at top of figure Lobulated form, with some margins covered by membranes The nucleus at the bottom of the figure contains large dark granules ×16,000

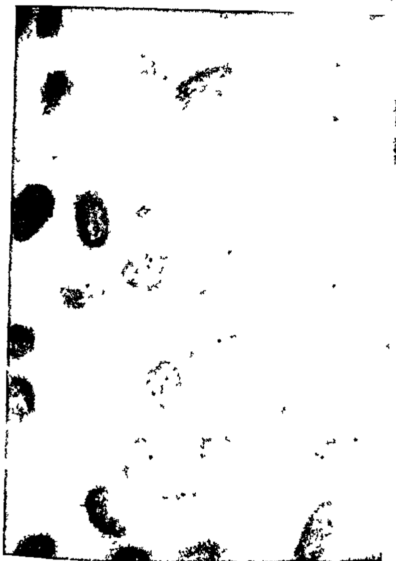
This granule does not seem to be unique for molluscum contagiosum, since it has been seen also by us in sections of the Shope fibroma elementary body, and seems to be present in Figures 6, 8, and 9 of Bernhard *et al*,[9] which show elementary bodies of the Shope fibroma virus It has not been seen in vaccinia



FIGURE 18 Swollen forms an l con lensed forms of the mature elementary body A por t on of sej ta is seen across the bottom ×21 000



FIGURE 19 Condensed f rms of the mature elementary l ody F ne fibr ls in a reticula network run l etween them Note the small dense granule in the wall of many of the elementary bod es ×22 000



F GURE 20 H gher magn ficat on of FIGURE 19 Note nner and outer doul le membranes of the elementa y l od es In some nstances a less dense ha o aj pea s about the lense granule w hin the wall ×84 000

Discussio i

Goodpasture and King[9] have described tl e extrus on of nucleoli into the cytoplasm and have demonstrated nucleolarl ke bod es in tl e cytoplasm early n the formation of the mollu cum body In the present stu ly relatively large

FIGURE 21 Mature elementary bodies One section is through the rectangular plane (*arrow*) ×84 000

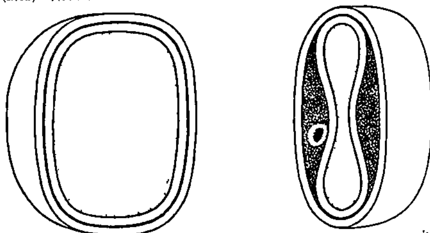


FIGURE 22 Diagram of the proposed structure of the condensed form of the mature elementary body Section through rectangular plane at left and normal to rectangular plane at right

bodies (FIGURES 6 and 7) were found in the cytoplasm, seemingly associated with early changes in the infected cell These were the only structures of a size that might appear as extruded nucleoli in the light microscope They have a distinct membrane that a nucleolus does not have, but this could be

part of the nuclear membrane added as the material passed into the cytoplasm This membrane, however, is coarser than the nuclear membrane and when the unit membranes are seen, these are denser than those in the nuclear membrane Their contents at times resemble what might be seen within some nucleoli or within the nucleoplasm Their membranes are coarser and their contents different from those of mitochondria Although they were first recognized as large bodies they seem to be related to similar smaller structures (FIGURE 6) Electronmicroscopically, their origin is obscure, and there is no strong evidence that they arise from nucleoli or mitochondria

The early inclusion is composed of a reticular component and foci of a granular component It appears that the granular foci give rise to the elementary bodies and therefore would be clearly a viral plasma The reticular component however, may be a reaction of the cytoplasm to the presence of the virus

Van Rooyen[11] has shown that the mature elementary bodies are embedded in a jellylike matrix that is seen in the electron microscope as a very sparse reticulum between the elementary bodies This structure is consistent with that of a gel, and may be derived from the reticular ground substance of the inclusion

There is a question as to whether the large dense granules within the nucleoplasm are specific for the virus infection or merely a nonspecific result of nuclear degeneration The work of Dourmashkin *et al.*[6] would suggest that these granules are specific, since these investigators observed them in HeLa cells infected with the virus of molluscum contagiosum, but not in degenerating cells of the control cultures

From the present study it is suggested that the elementary bodies of molluscum contagiosum form from the granular foci in the inclusion bodies by the following process (FIGURE 12) Dense granules of the order of 250 Å in diameter appear within the small granular foci These dense granules increase in size and the small granules organize in a zone about each of the large dense granules Eventually each dense granule becomes a nucleoid surrounded by a circumscribed reticular plasma with or without a membrane, Type I After a membrane has developed, the more solid contents including the nucleoid, retract toward the center, leaving an electron optically clear zone between the central dense structure and the membrane The nucleoid disappears, the membrane contracts, and the remaining particle, Type II, "germinates" The contents grow from the ruptured membrane, possibly fuse with the contents of other "germinating" particles, and grow into a large lobulated mass Type III This mass segments into the primordial elementary bodies, each of which is

e

e

a

t

viroplasm differentiates into two symmetrical shells [illegible] Type IV The outer membrane becomes double and an inner double membrane forms about the central area Thus it is proposed that this may be the sequence by which the mature elementary body is evolved

The four general types of structure preceding the mature elementary body are never seen within the granular component of the ground substance of the

small dense *granule* has not been seen in the nucleoid-containing body, Type I the ' germinating" structure, or the lobulated structure, Type III

As noted previously, the mature elementary body may be swollen as the result *of the* distention of the space within the inner membrane or it may be *in a condensed* form with the inner membrane collapsed In section, the *swollen forms* are slightly elliptical, with the long axis parallel to the thickened portion of the wall They may reach a diameter of 340 mμ as against a maximum diameter of 230 mμ for the condensed forms

Although the majority of the first formed elementary bodies appear to be swollen and those in the older inclusions near the surface of the lesion to be condensed, this may be the result of immediate environment rather than age, or it may be a combination of both environment and age

Certainly, Goodpasture and King's[3] suggestion that ' the main factor in the hyalinization of the intracellular mass is desiccation" holds true not only for the jellylike material surrounding the elementary bodies, but also for the elementary bodies themselves The absence of swollen forms in the old inclusions at the surface of the lesion and their abundance toward the base of the lesion would suggest strongly that the swollen forms had shrunk, indicating that at least the outer membrane must be quite elastic

Summary

The early inclusion body of molluscum contagiosum is composed of two types of ground substance, one is reticular and the other, occurring in focal areas, is granular This material distends the cell and pushes aside cell organelles The ground substance of the inclusion continues to increase, particularly the granular component At the same time, foci of mature elementary bodies are formed apparently at the expense of the granular component Eventually these foci almost completely fill the cell and divide the ground substance into septa

Tentatively, this process of elementary body formation begins with the appearance of dense granules of the order of 250 Å in diameter within the small granular foci These granules increase in size, and the small granules of the ground substance organize in a zone about each of the larger dense granules Eventually each dense granule becomes a nucleoid surrounded by a circumscribed reticular plasma with or without a membrane The nucleoid disappears and the remaining particle "germinates," forming a lobulated mass This mass segments into the primordial elementary bodies, each of which eventually is surrounded by a single membrane develops a dense granule and differentiates into a mature elementary body

The mature elementary body is found in two forms, one distended and the other condensed When condensed its shape is that of a rectangular body that appears as an ellipse when sectioned normal to the rectangular plane An outer and an inner membrane are separated by relatively homogeneous wall

mater al that increases in thickness as the central are t f
vex surfaces of the elementary body are approa h
inner membrane surrounds a potential space th t
distended form of the elementary body

W thin each mature elementary body is a den e
110 Å Th s granule is also present in some of he e
served in the ground substance of the early inclu
older inclus on It occurs in the Shope fibroma v r
in vaccinia

Acknowledgments

I thank Eugene Van Scott and Richard Reinert t the Nat onal Cancer Institute for supplying samples of molluscum les on and Bolivar Lloyd al o of the Nat onal Cancer Institute for a pellet of the Shope fibroma elementary bod es

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CYTOPATHOLOGY OF CANINE DISTEMPER STUDIED BY THE USE OF FLUORESCENT ANTIBODIES*

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Canine distemper is a highly infectious disease caused by a filterable virus.[1,2] It is a common malady of dogs, but it also affects foxes, wolves, ferrets, mink, skunks, and raccoons. The virus has been grown in the chorioallantoic membrane of developing chicken embryos[4,5] and is transmissible by intracerebral inoculations to suckling mice and hamsters.[6,7]

Canine distemper is of interest not only because of its importance to domestic and fur bearing animals, but also because there is accumulating evidence to show that the virus of this disease may be related in some manner to infection in man. The similarity of certain aspects of the tissue alterations between canine distemper and fatal cases of measles (rubeola) of children has been pointed out, most notably the proliferation of the lining cells of the pulmonary alveoli with the formation of multinucleate giant cells and the presence of both intranuclear and intracytoplasmic eosinophilic virus inclusion bodies.[8,9]

A similarity in antigenicity between distemper and a fairly ubiquitous infection conferring immunity in human beings has been suggested because of a rise in quantity from infancy to early childhood of a substance capable of neutralizing distemper virus as titered in chicken embryos.[10]

More recently, experiments conducted by culturing the virus of canine distemper in suckling mice and neutralizing with the measles antisera has shown an immunological relationship between these two diseases.[6] The transmission of measles to dogs has recently been reported[11] and one experiment, at least, has shown that distemper virus inoculated into a man survived for a period of six days.[12] It therefore appears that study of canine distemper is of importance not only because of its value to health of dogs and other animals, but because of its relationship to measles or some other, as yet unidentified, disease of man. Of equal importance, from the standpoint of cytology, is the extreme polytrophism of canine distemper. Because of this, distemper presents itself as does no other disease, as a model for investigation of the effects of such a virus.

Because of slight or aspecific gross changes in canine distemper, supposedly little specific histopathological alteration, and its value in presumptive diagnosis, considerable attention has been centered on the inclusion body. The presence of distinctive bodies in the disease was first reported in the neurons.[13,14] Soon their presence in ependymal cells and in bronchiolar and conjunctival epithelium, as well as in the neurons, was observed.[15] These inclusions were seen subsequently by many observers and the demonstration of inclusion bodies in tissue sections or smears from various epithelial surfaces came to be used as a diagnostic procedure. Due to this emphasis on the epithelial cell or brain as a site for inclusions and the respiratory or cutaneous nature of many of the signs of the disease, there has been a tendency to ascribe

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primarily epithelial invasive powers to the disease However evidence to the contrary has been reported by various observers who found characteristic inclusion bodies in lymphocytes, reticuloendothelial cells endothelial cells and, probably, other locations[16] In fact, as emphasized below polytrophism is the most striking characteristic of canine distemper



FIGURE 1 Photomicrograph of fluorescent antibody preparation of the gastric fundus showing the presence of specific antigenic inclusion bodies in the epithelial cells of the gastric tubuli (A) and within cells of the interstitium (B)

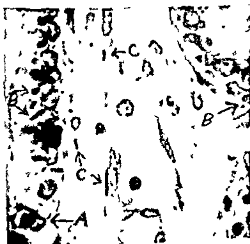


FIGURE 2 Photomicrograph of paraffin section through gastric mucosa at the level of the necks of the glands showing intranuclear inclusion (A) cytoplasmic inclusions in epithelial cells (B) and inclusions in the processes of fibroblasts (C) Hematoxylin and eosin stain

(6) Lymph nodes and spleen lymphocytes, reticuloendothelial cells leukocytes in abscesses

(7) Central nervous system neurons and their processes, macroglia ependymal cells, and endothelium of blood vessels

In sections studied by means of fluorescent antibody, antigenic material is seen in granules or masses in both the cytoplasm and nucleus of the affected

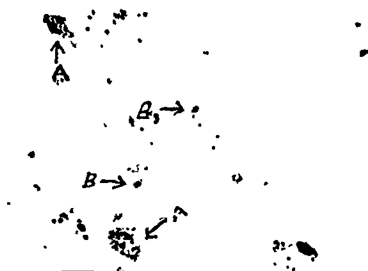


FIGURE 5 Photomicrograph of a fluorescent antibody preparation of the cerebellar peduncle showing specifically fluorescing distemper inclusion bodies in neurons (A) and macroglia (B)



FIGURE 6 Paraffin section of cerebellar peduncle showing hyperplastic vascular endothelium containing cytoplasmic inclusions of canine distemper Hematoxylin and eosin stain

cells which correspond generally to the size, shape, and distribution of distemper inclusion as seen in paraffin sections While the material in most locations is nearly always particulate, in the early stage of the invasion it occurs diffusely in the cytoplasm of the reticulum cells and the lymphocytes of the lymphoid tissue Even in these locations, it later assumes a particulate character Within the cytoplasm the masses vary in size from minute granules to bodies as large as the nuclei of the affected cells Intranuclear masses are usually single and of large size

Most of the body tissues excepting muscle bone and cartilage may be affected The specifically fluorescing masses occur even in leukocytes in the



FIGURE 7 Fluorescent antibody preparation of cross section of the neck of a hair follicle showing specifically fluorescent distemper inclusion bodies in the surrounding epithelial cells (A) Autofluorescence is seen in the hair shaft and in keratotic debris (B and C)

peripheral blood and in phagocytic cells participating primarily in other reaction for example, foreign body giant cells

The lesions of canine distemper are rarely diffuse, but seem to occur in disseminated foci in which the inclusions lie in fairly contiguous cells The affected ... more often

are

occi

glar

blas

astrocytes, neurons and blood vascular endothelium may be involved in the same focus that often appears related to blood vessels (FIGURE 3)

In ferrets and in a few dogs the virus appears to have severe cytopathogenic

effect, the affected cells being quickly destroyed In the ferret this may be associated with infiltrations of *neutrophils* and other changes of acute inflammation Th e ac t nfl mm t

distemper infection in this an mal W

is manifested in the dog,

not to be accompanied l

especially notable in the stomach, where loss of the chief cells with sparing of the parietal cells sometimes occurs (FIGURE 4)



FIGURE 8 Photomicrograph of paraffin section of comparable portion of hair follicles as in FIGURE 7 showing hyperkeratosis and the formation of intrafollicular microabscesses

However, the presence of the virus usually appears to elicit only cellular hyperplasia and cells undergoing mitosis, despite the presence of cytoplasmic distemper inclusions, can be found (FIGURE 5) This increased activity leads to the piling up of cells in the pulmonary alveoli with formation of giant cells In the brain it produces hyperplasia of the ependymal cells and astrocytes sometimes with multinucleate forms and hyperplasia of blood vascular endothelium with apparently, the formation of new vessels (FIGURE 5) In the skin this stimulation to hyperplasia, marked hyperkeratosis and parakeratosis in the epithelium of the skin and adnexal glands is a characteristic picture of canine distemper (FIGURES 7 and 8)

Summary

Canine distemper affects Canidae and certain other Carnivora The virus is transmissible to chicken embryos, and suckling mice and hamsters There

is an mmunolog cal relationsh p to measle The mo t d n e feature of the cy opathogen city are (1) presence of d t nct ve n lu on bod e on a n ng virus ant gen in both the cytoplasm an l the nucle of affected ell and he ab y of the v rus to invade most of the cell typ e of the body

Refere ces

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2 D I D d
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9 Adams J M D T Imagava D L Ci ad ck F H M Rela onsh p of meas es and d stemper A M A J I 95
10 Karzo D T 19 5 Stud es on a neut al z ng sub found n man Ped at cs 16 809
11 R aza.tzeva N E 1956 Fxpe mental mea l s p Epidem ol Immunob ol 27 5
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PROPAGATION, CYTOPATHOGENICITY, AND HEMAGGLUTINATION-HEMADSORPTION OF SOME ARTHROPOD-BORNE VIRUSES IN TISSUE CULTURE

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Tissue culture methods have been employed for the cultivation of arthropod-borne viruses for many years. One of the classic examples is the propagation of yellow fever virus by Theiler and his associates[1,2] in chick embryo tissue culture. At the present time more than seventy arthropod-borne viruses have been isolated. Certain of these agents are antigenically related and have been placed by Casals and Brown in distinct serologic groups A, B, and C[3,5] by means of hemagglutination-inhibition, complement fixation, and neutralization tests. In addition, while there is already indication of other groups, a large number of viruses still remain ungrouped.

Recent tissue culture investigations have been concerned chiefly with the study of some of these agents belonging to groups A and B.[6-14] During the past two years I have attempted the propagation of about 20 arthropod-borne viruses, some of which belong to groups A, B, and C, and a few of which are ungrouped. The aim has been to determine conditions under which yield of virus is high and the presence of virus easily and regularly detectable.

Methods of Study

Viruses

Table 1 lists the viruses used and the tissues that were employed for their growth. The following viruses were successfully propagated: Group A: Chikungunya,[7] Eastern equine encephalomyelitis (EEE), Mayaro,[15,19] Semliki Forest, Sindbis,[9] Western equine encephalomyelitis (WEE). Group B: Dengue Type 2, Japanese B encephalitis (JBE), Kyasanur Forest disease (KFD),[20] West Nile (WN). Group C:[4,5] Marituba (Be An 15), Oriboca (Be An 17), Apeu (Be An 848), Murutucu (Be An 974), Caraparu (Be An 3394). Other viruses: Bwamba,[2] Be H 5056, Be H 151, Be Ar 671, and Be Ar 278.

Many of these agents are well known. I shall therefore briefly describe only the unfamiliar viruses. Of Group A, Chikungunya virus, isolated from arthropods and man in East and South Africa, causes a denguelike disease. Mayaro virus, isolated in Bolivia, Brazil, and Trinidad, produces a systemic disease in man. Of

India is s

virus. In

disease. Group C is composed of six viruses that have been isolated i[illegible] d[illegible]se[illegible]se in

Belém area of the Amazon region. I

man. Of the other viruses, Bwamb

produces a system c disease in man.

were isolated in Belem by O. R. Causey. As yet they are not described

Cell Cultures

As growth of virus may be dependent on the type of cell used, chick embryo cells as well as several different human cell lines were chosen Cultures of whole, 9 day-old chick embryos were prepared by the method described by Dulbecco [22] The mammalian cells were maintained as described in TABLE 2 In the course of preliminary experiments, it was observed that the addition of tryptose phosphate broth (TPB)[23] to the growth medium apparently enhanced

TABLE 1
VIRUSES CELL SYSTEMS

Group	Virus	Chick embryo		HeLa TPB		S₁ HeLa		Detroit-6		Human embryonic intestine	
		I*	CPE†	I	CPE	I	CPE	I	CPE	I	CPE
A	Chikungunya	0	0	+	+	—	—	—	—	—	—
A	EEE	+	+	—	—	—	—	—	—	—	—
A	Mayaro, Tr 4675	0	0	+	+	+	+	—	—	—	—
A	Mayaro Tr 15537	+	0	+	+	+	+	—	—	—	—
A	Semliki Forest	+	+	—	—	—	—	—	—	—	—
A	Sindbis	+	+	—	—	—	—	—	—	—	—
A	WEE	+	+	—	—	—	—	—	—	—	—
B	Dengue Type II	0	0	+	0	+	+	—	—	—	—
B	JBE	+	0	+	+	—	—	—	—	—	—
B	KTD	+	0	+	+	+	+	+	+	+	+
B	WN	+	+	—	—	—	—	+	+	—	—
C	Marituba (Be An 15)	0	0	+	+	—	—	—	—	—	—
C	Oriboca (Be An 17)	+	0	+	+	—	—	—	—	—	—
C	Apeu (Be An 848)	0	0	+	+	+	0	—	—	—	—
C	Murutucu (Be An 974)	0	0	+	+	—	—	—	—	—	—
C	Caraparu (Be An 3394)	0	0	+	+	+	0	—	—	—	—
Other	Bwamba	+	+	—	—	—	—	—	—	—	—
Other	Be H 5056	0	0	+	0	0	0	—	—	—	—
Other	Be H 151	0	0	+	0	+	0	—	—	—	—
Other	Be Ar 671	+	0	+	0	0	?	—	—	—	—
Other	Be Ar 278	+	0	0	0	0	6	—	—	—	—

* Infectivity
† Cytopathogenic effect
Key + test for infectivity positive or cytopathogenic effect present 0, no viral multiplication or no cytopathogenic effect —, not tested

the rate of cellular multiplication of HeLa cells Our HeLa TPB cell line has undergone more than 180 transfers and has proved especially useful for the growth of some of the viruses For all studies employing mammalian cells, a maintenance medium consisting of 97 per cent of Eagle's medium[24] and 3 per cent of calf serum supplemented with antibiotics (100 units of penicillin, 100 μg of streptomycin, and 25 μg of mycostatin per 1 ml) was introduced before viral inoculation

Propagation and Cytopathogenicity

As can be seen from TABLE 1, every virus tested multiplied in at least one of the cell systems and often in more than one Presence of virus was detected by intracerebral inoculation of mice with the fluid phase of infected tissue cultures

TABLE 2
TISSUE CULTURE SYSTEMS

Cell culture	Source	H %	CEE %	Growth fluid HOS %	E %	HAS %	TPB %	Transfer intervals (days)	No of cells per tube
Chick embryo		90	5	5					5×10^6
HeLa TPB	Div of Laboratories and Research Albany N Y	65				20	15	7	2×10^6
S_3 HeLa	T T Puck via A Moore Sloan Kettering Institute New York N Y				80	20		7	2×10^6
Detroit 6	A Moore Sloan Kettering Institute				80	20		7	2×10^5
Human embryonic intestine	G Henle via Div of Laboratories and Research Albany N Y				80	20		7	2×10^5

* Containing 100 units of penicillin 100 µg of streptomycin and 25 µg of mycostatin per ml

Key H Hanks balanced salt solution adjusted to pH 7 4 to 7 6 CEE chick embryo extract HOS horse serum E Eagle s medium HAS human adult serum TPB tryptose phosphate broth

TABLE 3
CYTOPATHOGENIC EFFECT IN CHICK EMBRYO TISSUE CULTURE

Group	Virus	Titer* inoculum mouse brain (10^x c) LD50 (1 ml)	CPD50 (1 ml)	Number of passages	CPE†	Titer* current passage (fluid phase) LD50 (1 ml)	CPD (1 ml)
A	EEE	10 3	9 7	25	3+	8 0	8 0
	Semliki Forest	9 0	8 5	6	3+	7 8	8 7
	Sindbis‡	7 2	7 0	29	4+	7 4	8 0
	WEE	9 2	9 3	5	3+	8 8	8 5
B	West Nile‡	9 1	5 5	31	3+	7 8	8 3
Other virus	Bwamba‡	7 3	2 5	25	3+	5 2	5 3

TABLE 3 and TABLES 4 to 9
and granulation of scattered cells
f cells 4+, as above but most

The exceptions

al conditions for
TABLE 3
ruses that
PE) No
E viruses

WN and Bwamba viruses became well established only after several transfers Onset of cellular injury was rapid for group A and usually well established by the second day In group B, changes were first noticeable between the second and third days postinoculation, while Bwamba virus was even slower in that well-defined focal necrotic areas of cells (microplaques) preceded diffuse involvement of the monolayer All agents discussed in TABLE 3 were easily propagated when inoculated into freshly seeded tubes containing a massive cell inoculum of 5×10^6 cells/ml

With the viruses listed in TABLE 3, CPE in chick embryo tissue cultures was distinct and represented a reliable criterion for viral multiplication The specificity of the CPE was ascertained by neutralization tests using immune monkey sera The last two columns of TABLE 3 show that CPE was just as reliable as the production of deaths in mice for indicating the presence of virus Titer values in this and subsequent tables have been converted to values corresponding to 1 ml of test material

TABLE 4
PLAQUES CHICK EMBRYO TISSUE CULTURE

Group	Virus	Inoculum	Titer LD50 1 ml	Titer CPD50 1 ml	Titer PFU 1 ml	Days	Boundary of plaque	Diameter (mm)
A	WEE	Mouse brain	10 1	9 5	4×10^8	3	Sharp	6
A	WEE	Fluid phase 6th tissue culture passage		7 0	4×10^7	2	Sharp	6
A	Semliki Forest	Fluid phase 2nd tissue culture passage	8 1		1×10^5	3	Sharp	6
B	West Nile	Mouse brain	9 0	6 6	8×10^8	5	Sharp	3 4
Other virus	Bwamba	Fluid phase 12th tissue culture passage	5 8	5 5	2×10^3	8	Sharp	3

Assuming that the ability of a virus to produce cellular necrosis or injury is a prerequisite for the formation of plaques WEE Semliki Forest WN and Bwamba viruses were studied in chick embryo cells by Dulbecco's plaque technique [22] The results are shown in TABLE 4 Plaques with sharp boundaries were obtained either with infected mouse brain suspensions or with the infectious fluid phase of tissue cultures (1 ml of virus dilution per 150 ml bottle one hour adsorption at 37° C)

10^4 infectious mouse brain doses) were employed CPE occurred in all instances as of the very first passage and was constant thereafter With the exceptions of JBE virus and KFD virus complete destruction of monolayers was observed Successive passages were carried out as soon as slight cellular

changes became visible, at short intervals of 3 to 4 days Microscopically distinct areas of focal cellular necrosis (microplaques) progressing to complete involvement of the monolayer were characteristic for Chikungunya, Mayaro, and viruses of group C This indicates that Dulbecco's plaque technique in all probability, could be used successfully with these agents FIGURES 1 to 4 show Giemsa stained[45] clonal S_3 HeLa cells as well as HeLa TPB cells inoculated with Mayaro virus, strain Tr 15537, and Oriboca virus, respectively *The focal nature of the initial lesion is evident especially from* FIGURE 3 In cultures infected with the newly discovered KFD virus and with JBE virus, injury was more diffuse Early lesions consisted of rounded and granulated cells scattered throughout the monolayer Plaques with poorly defined edges

TABLE 5
CYTOPATHOGENIC EFFECT IN MAMMALIAN CELLS

Human cell line	Group	Virus	Log of infectious mouse brain doses inoculated at start of experiment	Number of passages	CPE*	Titer of fluid phase of infected tissue cultures	
						LD50 (1 ml)	CPD50 (1 ml)
S_3-HeLa	A	Mayaro	5 1	16	4+	6 3	7 2
S_3-HeLa	A	May Tr 15537	6 2	16	4+	6 8	7 4
S_3 HeLa	B	Kyasanur Forest Disease	6 7	25	3+	8 0	7 8
HeLa TPB	A	Chikungunya	4 6	10	4+	6 0	7 1
HeLa TPB	B	Kyasanur Forest Disease	6 7	50	3+	8 0	8 5
HeLa TPB	B	*Japanese B encephalitis*	4 7	6	2+	5 8	6 0
HeLa TPB	C	Marituba (Be An 15)	5 0	10	3+	5 9	6 3
HeLa TPB	C	Oriboca (Be An 17)	6 5	10	4+	6 5	7 0
HeLa TPB	C	Murutucu (Be An 974)	6 5	10	4+	5 9	7 0

* CPE see footnote, TABLE 3

have been described by Bhatt and Work[14] in monkey kidney cultures inoculated with JBE virus

Hemagglutination Hemadsorption

During experiments designed to propagate KFD virus in chick embryo tissue cultures it was found that, although a large amount of virus was produced as [illegible] the search

y mouse

xplored

hrocytes

[illegible]

as a means of virus detection in tissue cultures inoculated [illegible]

mumps and croup associated (CA) viruses. Their findings encouraged us to search for a similar phenomenon in cultures inoculated with KFD virus. We were successful only when pH conditions similar to those described for the conventional hemagglutination (HA) and hemagglutination inhibition (HI) test by Clarke and Casals[31] were maintained. To achieve this we used the same chemical reagents and, insofar as possible, the same techniques that they recommend.[31] At the present time a positive hemagglutination hemadsorp-



FIGURE 1. Clonal S3 HeLa cells inoculated with Mayaro virus, strain Tr 15537, harvested 24 hours after inoculation and stained by Giemsa's method. Note contraction, rounding and granulation of cells. Increase in cytoplasmic basophilia (diffuse or granulated). ×5 0

tion test in tissue culture (THA) has been obtained for the following viruses: WEE, Sindbis, KFD, and WN.

The THA test for KFD virus is routinely performed as follows. The fluid phase of infected and uninfected cultures is removed. The cellular monolayer is washed once with 1 ml. of Hanks' balanced salt solution adjusted to pH 7.4 to 7.6. The washing fluid is discarded and borate saline (pH 9.0) is added, 0.2 ml. per culture. After incubation at 37° C. for 30 min., goose erythrocytes suspended in virus adjusting diluent to give a final pH of 6.4 (considered opti-

mal for KFD virus) are introduced 0.2 ml per culture and mixed well. For optimal results the optical density of the red cell suspension is higher than the one used in the HA or HI test and corresponds roughly to a dilution 1:11 of the standardized cell stock[31] or approximately is equal to a 1 per cent suspension of packed cells and has an optical density of 1.8. Following a second incubation of 1 hour at 4° C tubes are drained for 5 min and the test is read immediately.

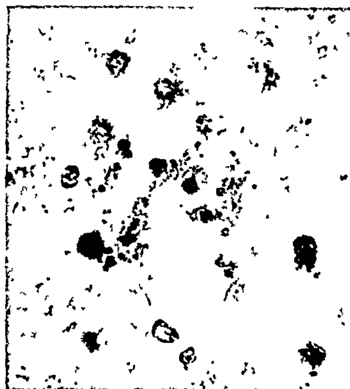
FIGURES 5 to 7 representing unstained clonal S_3 HeLa cells infected with the



FIGURE 2. Clonal S_3 HeLa cells uninoculated stained by Giemsa's method. Monolayer intact. ×570.

forty-ninth passage of KFD virus show in addition to CPE the characteristic findings of the THA test. The specific sign for presence of viral hemagglutinin is an end to end agglutination of the nucleated goose erythrocytes. From 3 to 12 red cells are *linked together longitudinally*. Such a unique arrangement of red cells has been observed only in infected cultures and is not seen in uninfected preparations. This chain formation can be inhibited specifically by ... In a positive THA test the ... o other chains appearing then ... ome attached around isolated

cells or groups of cells horseshoe formation or rosette formation occurs At



cells or to tube wall The length of the red cell chains depends on the optical density of the red cell suspension and on the amount of viral hemagglutinin If the optical density of the added red cell suspension is reduced to 0 75 a value used in the plate HA and HI test chains are shorter double and triple chains are rare and rosette formation is usually lacking Readings of THA tests have been based exclusively on chain formation In scoring the results the following system was used + chain formation (3 or more erythrocytes), ± occasional chains (2 to 3 erythrocytes) and 0 no chain formation To

tests it must be shown that uninoculated controls are completely free of chain formation.

The quantitative aspects of the THA test as a means of detecting virus multiplication were then tested as summarized in TABLE 6 and in FIGURE 8. KFD virus was propagated for many passages in chick embryo tissue cultures, HeLa TPB cells, clonal S_3-HeLa cells, Detroit 6 cells and human embryonic intestinal cells. Viral hemagglutinin was produced in all systems. Fluid phases of in-

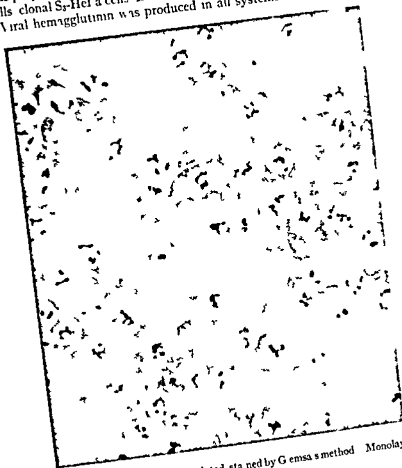


FIGURE 4. HeLa TPB cells uninoculated stained by Giemsa's method. Monolayer intact. ×570

fected cultures were titrated in mice and tissue cultures and LD_{50} titers were then compared with CPD_{50} (cytopathogenic effect doses$_{50}$), THD_{50} (tissue culture hemagglutination hemadsorption doses$_{50}$) and TID_{50} (tissue culture infectious doses$_{50}$). The latter figure was obtained by mouse infectivity tests with the undiluted fluid of cultures inoculated with different virus dilutions. The values listed in the two last columns of TABLE 6 indicate a good correlation between THD_{50} and TID_{50}. This fact was of great importance for the cultivation of KFD virus in chick embryo tissue cultures where CPE was absent in

spite of a very high TID_{50}. Whenever viral hemagglutinin was present, infectious virus was present, too. As another approach to correlating THA and infectivity, a very small amount of KFD virus, about 100 THD_{50} (forty-fourth passage of KFD virus in chick embryo suspension culture), was inoculated into freshly seeded tubes containing 5×10^{4} chick embryo cells/ml, thus ensuring the greatest opportunity for contact between the cell population and the small virus inoculum. Appearance of infectious virus in the fluid phase was studied at different times by mouse infectivity tests and THA tests in

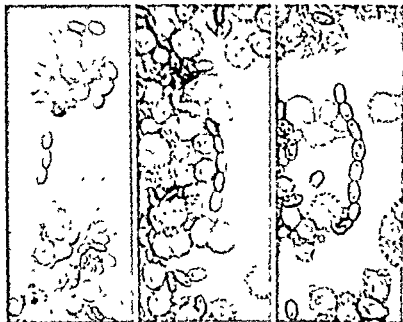


FIGURE 5. Cl[illegible]nal [illegible] HeLa cells inoculated with KFD virus, harvested 48 hours after inoculation, unstained. [illegible] hains of 3, 5, and 7 longitudinally agglutinated nucleated erythrocytes. Consi[illegible]le [illegible] pathogenic effect. ×355

chick embryo tissu[illegible]ltures (FIGURE 8). The slopes of the curves are very similar, indicating th[illegible] THA test is as valuable a means for the detection of virus as the mouse [illegible] technique.

[illegible] Antibodies in Tissue Culture

Neutralization test [illegible] Murutucu virus Be An 974 (Group C). The ability of Murutucu virus [illegible] HeLa TPB cells was utilized in the assay of antibodies in a hyperimmun[illegible] mouse serum. The results of the experiment are shown in TABLE 7. [illegible] pooled sera of uninoculated and of vaccinated mice were tested at the sa[illegible]. Equal amounts of undiluted serum mixed with varied concentrati[illegible] virus suspensions (tenth passage of Murutucu



FIGURE 6. Clonal S_3-HeLa cells inoculated with KFD virus harvested 48 hours after inoculation, unstained. Note chains of red cells attached to single infected cells. Horseshoe formation. ×355.

FIGURE 7. Clonal S_3 HeLa cells uninoculated control preparation, unstained. THA test negative. A few red cells trapped mechanically. ×355.

virus, Be An 974 in HeLa TPB cells fluid phase) were incubated at 37° C for 1 hour, then inoculated into mice intracerebrally in 0 03 ml amounts and into tissue cultures in 0 1 ml amounts. Results were clear cut. In both cases complete neutralization was obtained with all dilutions tested

TABLE 6
KYASANUR FOREST DISEASE COMPARISON OF LD$_{50}$ IN MICE TCPD$_{50}$ THD$_{50}$* AND TID$_{50}$†

Culture	Passage	LD$_{50}$ (1 ml)	TCPD$_{50}$ (1 ml)	THD$_{50}$ (1 ml)	TID$_{50}$ (1 ml)
Chick embryo	5	>7 0	1 5	7 5	7 5
Chick embryo	15	7 3	<1	8 5	8 4
Chick embryo	20	7 3	<1	8 3	8 3
Chick embryo	37	7 5	<1	8 1	8 1
HeLa TPB	5	7 8	5 8	6 5	6 5
HeLa TPB	10	8 3	7 2	8 5	8 5
S_3-HeLa	5	7 8	6 5	6 5	6 5
S_3 HeLa	10	8 0	7 2	7 3	7 3
S_3 HeLa	15	8 7	8 0	8 0	8 0
S_3-HeLa	25	8 0	7 8	7 8	7 8
Detroit 6	20	8 8	Incomplete	7 2	7 3
Human embryo intestine	10	7 2	7 5	7 5	7 3

* THD$_{50}$ tissue culture hemagglutinin hemadsorption D$_{50}$
† TID$_{50}$ tissue culture infectious D$_{50}$

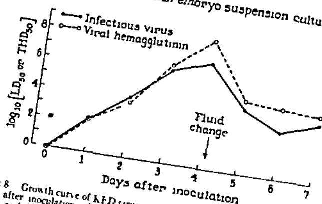


FIGURE 8 Growth curve of KFD virus in chick embryo tissue culture. Medium changed four days after inoculation. In this figure titers are expressed as log of D$_{50}$ per 1 ml (●——●) and log THD$_{50}$ per 1 ml (O - - - O) of pooled fluids of three tubes. The asterisk indicates the titer of the original inoculum. Pooled fluids were titrated in adult mice and in chick embryo tissue cultures. LD$_{50}$ 10^0 or THD$_{50}$ 10^0 indicates no detectable virus

Neutralization test (TH 1) KFD virus (Group B) Sera of young adult hamsters inoculated intraperitoneally with about 10^5 infectious doses of KFD virus were collected 1 month postinoculation and tested for neutralizing antibodies. Sera of uninoculated hamsters were collected and tested at the same time. The test was carried out as follows undiluted sera were mixed with

100 LD_{50} of KFD virus (10 per cent infused mouse brain diluted 10^{-5}) and incubated for 1 hour at 37° C The mixtures were then inoculated into chick embryo tissue cultures (0 1 ml), as well as intracerebrally into adult mice (0 03 ml) Six tubes or six mice, respectively, were used for each mixture *Due to occurrence of nonspecific deaths, the number of mice was less than 6 in* some instances Parallel titrations were performed in both tissue cultures and mice at the same time Mice were checked for the occurrence of deaths every day for 15 days and the average survival time (AST) calculated Chick embryo tissue cultures were tested for the presence of viral hemagglutinins by the THA test on the fifth day after inoculation Results of this experiment are shown in TABLE 8 Viral hemagglutinin was detected in all tubes inoculated with mixtures of virus and sera devoid of antibodies In tubes inoculated with mixtures of virus and sera from immune animals, the virus was neutralized and THA tests were negative There were no discrepancies between the tissue culture and mouse neutralization tests, the former evaluated on the fifth day as compared to the latter, which was terminated 15 days postinoculation

TABLE 7
GROUP C NEUTRALIZATION OF MURUTUCU VIRUS

Virus* dilution	Normal mouse serum		Hyperimmune mouse serum	
	Infant mice (intracerebrally)	HeLa TPB cells	Infant mice (intracerebrally)	HeLa TPB cells
10^{-2}	7/7	3/3	0/8	0/3
10^{-3}	7/7	3/3	0/8	0/3
10^{-4}	7/7	3/3	0/8	0/3
10^{-5}	1/7	2/3	0/8	0/3
10^{-6}	0/7	0/3	0/8	0/3
Titer†	6 1	6 3	<2	<2

* Source of virus tenth passage of Murutucu virus in HeLa TPB cells fluid phase
† Expressed as $\log_{10}$/1 ml of test material

Hemagglutination hemadsorption inhibition test (THI) KFD virus (Group B) In these experiments sera of 6 laboratory workers, of 2 rabbits immunized against KFD virus, as well as of 1 normal rabbit were tested by conventional HI test as well as by the THI test All sera were treated with kaolin and adsorbed with goose erythrocytes [31] The same conditions were necessary for success in both methods Five days postinoculation of 100 THD_{50} into chick embryo tissue cultures, a *THI test was performed as follows* Treated sera, diluted 1 10 in borate saline (*p*H 9 0) were added to washed cultures, 0 2 ml [illegible] 30 m n at 37° C the red cells were added

C , tubes

TABLE 9 shows a comparison of results obtained by the HI test with those from the

TABLE 8

GROUP B NEUTRALIZATION OF KFD VIRUS IN CHICK EMBRYO TISSUE CULTURE (THA) AND BY INTRACEREBRAL MOUSE TEST

Sera	Virus dilution (10% mouse brain)	THA test*							Intracerebral mouse test			
		No of tubes						Result	Died	Survived	AST (days)	Result
		1	2	3	4	5	6					
Hamster 1 N	10^{-6}	+	+	+	+	+	+	6/6	6	0	6 5	Neg
2 N	10^{-6}	+	+	+	+	+	+	6/6	6	0	6 3	Neg
3 N	10^{-6}	+	+	+	+	+	+	6/6	6	0	7 2	Neg
4 N	10^{-6}	+	+	+	+	+	+	6/6	4	0	6 5	Neg
5 N	10^{-6}	+	+	+	+	+	+	6/6	2	0	7 0	Neg
6 Imm	10^{-6}	0	0	0	0	0	0	0/6	1	4	14 0	Pos
7 Imm	10^{-6}	0	0	0	0	0	0	0/6	1	5	13 0	Pos
8 Imm	10^{-6}	0	0	0	0	0	0	0/6	0	6	15 0	Pos
9 Imm	10^{-6}	0	0	0	0	0	0	0/6	1	4	13 2	Pos
10 Imm	10^{-6}	0	0	0	0	0	0	0/6	1	4	13 6	Pos
Control		0	0	0	0	0	0	0/6	ND	ND	ND	
Virus titration	10^{-5}	+	+	+	+	+	+	6/6	5	0	6 6	
	10^{-6}	+	+	+	+	+	+	6/6	4	0	7 7	
	10^{-7}	+	+	+	0	+	0	4/6	4	1	10 0	
	10^{-8}	0	0	+	0	0	0	1/6	0	5	15 0	
	Titer † THD_{50} = 8 4								LD_{50} = 8 9			

* Scoring explained in test.
† Expressed as $\log_{10}$/1 ml. of test material

TABLE 9

GROUP B THI TEST WITH KFD VIRUS IN CHICK EMBRYO TISSUE CULTURE

Sera	History	Tissue culture* THI test	Plate† HI test
Human, W R	No vaccine	0	0
Human, C M	RSSE vaccine	4	3
Human, N D	RSSE vaccine	0	0
Human, S M B	RSSE vaccine	3	0
Human, D N D	Laboratory infection with KFD virus	8+‡	4
Human, R C	Laboratory infection with KFD virus	8+	7
Rabbit, No 1	KFD—mouse brain susp	5	7
Rabbit No 2	KFD—mouse brain susp	6	8
Rabbit, No 3	Uninoculated	0	0

* THD_{50}, $10^{-4\ 8}$/1 ml on day of test
† Plate test performed with 8 units of hemagglutinin
‡ End point not reached.

the immunizing agent The only discrepancy occurred when sera of laboratory workers vaccinated with formalized Russian Spring Summer encephalitis (RSSE) virus were tested for HI antibodies against KFD As has been pointed out, KFD virus is serologically closely related to the RSSE virus Also it must be remembered that in the HI test 8 units of hemagglutinin were used as contrasted with 1 5 × 10^5 THD_{50}/ml viral hemagglutinin doses determined

on the day on which the THI test was carried out Since there is no reason to assume that the amount of antigen is the same in such different tests as the HI and THI, it is perhaps, remarkable that the degree of correlation was so close

Summary

Arthropod borne viruses are easily propagated in either chick embryo tissue cultures or mammalian cell lines

Viral infectivity can be measured by direct observation of cellular alteration (CPE) or by production of viral hemagglutinin as measured in the tissue culture hemagglutination hemadsorption (THA) test

Assays of antibodies either by neutralization tests or by tissue culture hemagglutination hemadsorption inhibition (THI) tests appear to correlate reasonably well with results obtained in mouse protection tests or HI tests

Tissue culture appears to be an effective device for the cultivation and assay of a large group of arthropod borne viruses and the detection of their antibodies

Acknowledgments

I acknowledge the helpful and constructive criticism given by J Casals D H Clarke R Morison Max Theiler, and Loring Whitman A Oya was responsible for preliminary experiments pertaining to hemagglutination hemadsorption technique R E Shope propagated several of the viruses The valuable technical assistance of W Rodriguez C Mullen and E Rodriguez is also gratefully acknowledged

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BIOCHEMICAL STUDIES OF VIRUS INFECTED CELLS*

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The orientation imposed by the subject of this monograph leads us to em phasize those asp[illegible]

[illegible] the ensuing chain of secondary reactions and the translation of molecular alterations to macrochanges together, comprise the scope of our interest Other papers in this monograph [illegible]
express pre-eminently how [illegible]
of any viral infection can be [illegible]
in general terms the over all pattern as it appears in the light of present obser[illegible]

[illegible]

the chemistry of the virus has waited upon developments in tissue culture that allow first of all the important separation of the primary cytopathology from the inflammatory and proliferative effects of a secondary nature An equally important result of the tissue culture technique is the ability to observe changes most easily by comparison of infected and normal cultures where, because of the completeness of infection one is really comparing normal and infected cells Only preliminary studies of the rate and nature of the initiation of infection allow one to proceed with assurance in this regard

A variety of techniques is available to limit the infection in a particular culture to a single sequence This is an essential for the correlation of viral synthesis with the observed cellular changes and for the determination of the natural order of these processes If classic methods of biochemistry are to be employed the use of mass cultures becomes mandatory and of greater value where infection is initiated synchronously in all cells since this provides for interpretation at the cellular level The few results already available empha size the value of complete time studies during the infectious cycle if we are to have confidence that important observations are not overlooked

Thus with present techniques it is possible to direct our observations to very uniform multicellular cultures in which nearly every cell is undergoing a single cycle of infection in an almost synchronous manner However what is desired are cultures in which all cells during their growth cycle are performing physiological and biochemical activities synchronously

Also prerequisite to a determination of effects whose cause is viral is a clear notion of the properties of the normal cell these properties must be definable and measurable

Inductive or stimulatory effects are best considered in resting cell systems

* The work reported in this paper was supported in part by a grant from The National Foundation New York N Y

wherein activities are only potentials and in which the environment is competent for the realization of the potentials Success in provoking a particular activity by virus or any other instrument establishes the competency of environment for this activity, failure only leaves the question unanswered

Inhibitory or blocking effects of virus, in contrast are best studied with cells in a dynamic state where the activities in question are proceeding Properties of cells manifest only in the dynamic state are elegantly followed in synchronized cultures or in single cells

Observations that fall in the first category of stimulatory or inductive effects will be considered first Many of the findings presented elsewhere in [illegible] monograph are appropriate here and could serve as examples [illegible] because I have greater familiarity with the details of the studies [illegible] H F Maassab, I shall emphasize these preferentially

Maassab reported that the composition of his control cultures during the experimental period remained constant when considered on a cellular basis Constant composition would result if a static situation existed in which no net synthesis of ribonucleic acid (RNA) or desoxyribonucleic acid (DNA) was occurring or equally as well if synthesis was proceeding rapidly in cells also undergoing completely asynchronous division

Careful study of cellular division in cultures of ordinary HeLa cells sustained in 90 per cent maintenance solution (Scherer, 1953) and 10 per cent equine serum indicates that the former situation prevails In the maintenance medium cellular increase does not occur, net synthesis of cytoplasmic RNA nuclear RNA, and DNA does not proceed at detectable levels although these components may be metabolically active as indicated by the considerable radioactive phosphate that they incorporate The cells, of course, are utilizing glucose, consuming oxygen, and producing acid Upon replacement of the maintenance medium with 90 per cent Eagle's medium (Eagle 1955) and 10 per cent equine serum, cellular division resumes the cells grow and increase in number At present it has not been determined whether the maintenance medium either is simply deficient or is inhibitory however, in either case, the effects are multiple, although possibly secondary to a primary or singular defect

When such cultures of resting cells, sustained in maintenance medium are infected with poliovirus, as Maassab has described, copious quantities of RNA and protein accumulate in the cytoplasm It follows therefore, that in the system precursors of RNA and protein and an appropriate source of energy are available In the resting cell these syntheses are potential, the environment is

[illegible] for the
[illegible] phase does not occur
[illegible] esis of DNA or nuclear
RNA, it is not possible to decide whether [illegible] nce medium is compatible with these reactions or not Conclusions cannot be drawn as to whet[illegible] the virus, on infecting the cell, is able to st[illegible] syntheses or ev[illegible]

can inhibit them The competence of the environment for DNA *synthesis* might be established by infecting the cell with some other virus (Newton and Stoker, 1958) or the question might be examined experimentally with cells in a growth medium As yet, these experiments have not been completed *It should be emphasized, however, that there are distinct* early metabolic, as well as morphologic, effects demonstrable in the nucleus of infected cells

So much for the basic description of the normal resting cell and its potential activities The next consideration is one of the viral effect that has been described the hyperdevelopment of cytoplasmic elements (Maassab *et al*, 1957, Loh *et al*, 1958)

The synthetic activity induced in the resting cell by the infection may not exceed that of the ordinary cell during the growth phase, particularly if we consider that the RNA and DNA of the daughter cell probably are formed only during a portion of the interphase and that the total generation time is only eighteen hours One need not assume that the virus increases the basic *potential of the cell*

The effect here of virus is conceived most simply (and this is speculation) as an uncoupling or disengaging action that releases the cytoplasmic reactions from obligate cross linkages with other major synthetic activities with which they are normally coordinated

The constant quality of [illegible] suggests the operation during cellular [illegible]

synthesis of various speci[illegible]

synthesis of one species of RNA could not proceed independently of others When conditions in a particular locus of the cell are suitable for RNA synthesis, all templates in the area function and, possibly by simple competition, duplicate *in fixed proportions*

Again, it is economical to propose that this normal coordinating principle operates after infection and that replication of the viral specific species of RNA triggers the development of other templates of RNA already present in the host cell

It is obvious that we do not understand the principle underlying the coordinated synthesis of the various RNA molecules in the ordinary cell and much less how the virus may participate under such a principle One clue may be the fact that the infectious cycle can be initiated by the isolated RNA of the poliovirus If we restrict our thinking to the nucleic acid it is tempting to propose that in the ordinary cell nucleic acid synthesis occurs only at those times when the nucleic acid is freed from the nucleoprotein Perhaps the simple presence of an active or free nucleic acid molecule in the metabolic milieu is sufficient to catalyze the dissociation of the resident nucleoproteins In one sense the action of the virus would be to condition certain areas of the *cell to* [illegible]

that was not virus and not destined *to be virus* but [illegible]

It is quite clear from experimental evidence (the quantities of materials involved, their distribution among the subcellular components, and the base

with adenovirus (Boyer *et al.*, 1957). The second point, regarding the cellular

plasm. This corresponds to fraction III, which we have found to increase in total protein content (Loh *et al.*, 1958).

At this point we should not fail to recall that well authenticated examples can be cited also in which there is the induction of synthesis of materials characteristic of infection, but of neither viral nor cellular nature. I refer to the proteins comprising the crystalline matrix found in cells of insects with polyhedral diseases (Bergold, 1953), the hemagglutinating protein of vaccinia infection (Chu, 1948), and the nonviral antigens found after phage infection of *Escherichia coli* (Watanabe, 1957). For these one would like to reserve some special functions in the viral developmental process. These may be of structural or, as with the T_2 bacteriophages, of metabolic significance (Flaks and Cohen, 1957).

A third class of materials, not to be ignored in preparing a balance sheet for infected cells, is that comprised by products resembling virus but lacking full biological activity. This includes all the well established soluble antigens and so-called incomplete virus forms. These are of such extensive and diverse natures that it would not serve our purpose to review them here.

It suffices to say that with poliovirus infection and, possibly, with the others cited, the first class of newly formed materials is quantitatively of the greatest significance and accounts for much of the histochemical visualization.

The central problem is: What is the biochemical basis of the cytopathogenic effect, how can any of the considerations just outlined be related to the central problem?

From a teleological viewpoint, there would seem to be value in the suppression of limited phases of cellular development under conditions restricting the full de

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With the HeLa cell two examples may be cited: the action of X irradiation

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ever, once unbalanced development has occurred in the presence of inhibitor, viability cannot be restored upon the addition of thymine; the change is ir-

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stimulated by the virus is out of phase with other normal activities of the cell The cytopathogenic effect might have its primary basis *not in the* synthesis of a small amount of foreign material, but in an unbalanced growth of the cell However, the final loss of morphologic integrity may result from the secondary accumulation of some particular substance Such a cytotoxic substance has been described in a preliminary publication (Ackermann *et al*, 1958*a*)

Few ideas are truly new, the concept that growth stimulation is primary to necrosis has been suggested before on purely morphologic evidence (Rivers 1928) In fact, some classifications of virus based upon pathology suggest a spectrum of agents those causing neoplasms, those producing hyperplasia and necrosis and finally, agents with pronounced necrotic effects.

The idea that the action of virus is fundamentally a stimulatory one would not be questioned in the case of carcinogenic viruses Of interest in this connection is the recent report that, under different environmental circumstances, the polyoma virus may produce either necrotic or proliferative cytopathogenic effects (Eddy *et al*, 1958) Likewise, the late Duran Reynals (1940) was much occupied with this dual nature of viruses It might be proposed that all viruses possess this fundamental capacity to uncouple limited metabolic and structural areas of cells different viruses, for example, those containing RNA or DNA, would affect different loci The carcinogenic viruses would stimulate such wide areas that the cell may survive, although considerably altered

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LOH [illegible]

RIVE[illegible]
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RELATIONSHIP OF VIRAL ANTIGENS TO INCLUSION BODIES

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The technique of labeling antibodies with fluorescein, as developed by Coons *et al*,[1,2] has become a very useful tool for immunohistochemical studies It has a particular advantage in the study of viral infections because the antigens may be localized precisely in an infected cell by this relatively simple procedure

The antibody molecules in a specific serum can be labeled with fluorescein and used as a direct histochemical stain for antigens Many viruses such as influenza, mumps, vaccinia, fowl plague, Egypt infectious canine hepatitis and canine distemper have been detected by this direct staining method in tissue sections, as well as in infected cells from tissue cultures On the other hand, unlabeled specific antiserum can be placed over tissue cells containing antigen The antibody globulin molecules are deposited on the sites of antigen After the excess of antibody has been washed away, the bound antibody molecules may be detected by fluorescein labeled specific antiglobulin

(4) the unlabeled serum can be diluted for antibody titrations Measles varicella, primary atypical pneumonia, herpes simplex poliomyelitis psittacosis and others have been successfully studied in this way

It is obvious that fluorescent antibody staining will help to elucidate the question of whether inclusion bodies represent aggregates of viral particles or are merely morphological residuum from viral infections Several viruses that produce classic inclusion bodies have been investigated

Infectious canine hepatitis is an acute disease of viral origin in dogs and is characterized by centrolobular necrosis of the liver Histologically, large intranuclear eosinophilic inclusion bodies are seen in the hepatic parenchymal cells, as well as in the endothelial cells of smaller veins, venous sinuses, and liver sinusoids The typical Type A inclusion bodies had been noted by Cowdry and Scott[3] in 1930 in two experimentally infected dogs However, the disease was first described under the present name by Rubarth[4] in 1947 Coffin *et al*,[5] using the direct method of fluorescent staining demonstrated that the intranuclear inclusions in experimentally infected dogs contained a

hematoxylin and eosin The nuclear membrane continued to contain viral antigen during the entire course of development of the inclusion bodies

Canine distemper is a common infectious disease in dogs, foxes, and weasels

The causative agent is a filtrable virus first discovered by Carré[6] and later isolated by Laidlaw and Dunkin[7] Inclusion bodies in various body tissues of infected animals have been demonstrated frequently by staining with hematoxylin and eosin, by Seller's method,[8] or by Shorr's method[9] When smears of urinary bladder epithelium from infected dogs were treated with specific fluorescein labeled anticanine distemper serum, numerous fluorescent masses were seen in the cytoplasm of the epithelial cells After the smears were studied under the fluorescent microscope and photomicrographs were taken, the same slides were then stained by Seller's or Shorr's method It was found that in

tory[11] However, smears stained by Shorr's method (S-3 stain) showed interesting differences The reddish inclusion bodies characteristic of distemper infection[9] usually did not contain viral antigen The fluorescent masses of viral antigens appeared faintly gray blue in the S 3 stains, and would often be passed unnoticed if they were not looked for specifically This suggests a ... f distemper inclusions During the early stage of develop-

ing on their stage of development

The problem of whether the intranuclear eosinophilic Type A inclusions in herpes simplex infections contain virus particles has aroused the interest of many investigators Cowdry[12] in 1930 found that the herpes inclusions did ... reaction Similarly Francis and Kurtz[13] concluded ... virus is not associated with

clusions contain the nucleoprotein intranuclear inclusions do not possess such nucleoprotein Gray and Scott,[16] in their differential centrifugation experiments in herpes infected chick embryo liver, demonstrated that significant amounts of infectious virus could be recovered from isolated nuclear fractions of infected liver during the early stage of infection while the nonnuclear fractions of virus increased as the infection progressed Morgan *et al*,[17] by electromicroscopy, suggested that the initial site of herpes virus development is in the cell nucleus and subsequently is released into the cytoplasm, where it becomes mature Using the fluorescent antibody and hematoxylin eosin staining methods, Lebrun[18] studied the course of the development of herpes simplex inclusions in infected tissue culture of human epidermoid carcinoma cells She observed that the detectable herpes antigen first appears as small fluorescent spherical spots in the nucleus, slowly increases in amount, and finally fills the whole nucleus At this stage the clear antigenic material is basophilic and does not correspond to the T... inclusions Specific antigenic material gradually diffuses into the cy... nd increases in amount At the same time the nucle... creases until it becomes very small or absent Cells at th... esence of typical Type A inclusions that contain little

From all the above data it is clear that, in the infected cells, herpes simplex virus multiplies in the nucleus, incorporates desoxyribose nucleoprotein [14, 15] assumes a central body 30 to 40 mμ in diameter with a single membrane [17] and is antigenic [18]. As the development progresses the virus is released from the nucleus to the cytoplasm and assumes a second outer membrane [17] leaving the [illegible]

bodies in brain smears or sections is the most helpful procedure beside actual isolation of the virus. Wolman and Behar[19] in following the development of Negri bodies by the Feulgen reaction and by studying phosphatase and cholinesterase activities suggested that the inclusions represent aggregates of virus particles that multiply up to a certain limit and then diminish in number thereby resembling the events in morphogenesis of herpes inclusions. Goldwasser and Kissling[20] have demonstrated that the Negri bodies in infected animal brains, as well as in salivary glands contain viral antigen. Although the studies were not carried out in staining with fluorescent antibody and other histological stains of identical cells the demonstration is sufficiently convincing because the fluorescent staining of the Negri bodies was inhibited by removal of the antibody with specific rabies virus. The authors suggested that this method may be useful in the diagnosis of Negri negative animals and in serologic assay of immunity in man or other animals undergoing rabies vaccination.

In summary the use of fluorescent antibody as an immunohistochemical stain has helped investigators to understand more about the problem of the relationship of viral antigens to inclusion bodies. In infectious canine hepatitis the intranuclear inclusions of this disease contain a high concentration of viral antigen that begins in the nuclear membrane and spreads to the interior of the nucleus. In canine distemper the inclusions may or may not contain viral antigen depending on the staining method used. In herpes simplex it is evident that viral antigen, presumably representing active virus is found in the inclusion bodies during the early stage of development. When the infection has run its course the virus is released from the intranuclear site of multiplication and leaves behind the eosinophilic inclusion as a cytological scar. More studies on the Negri bodies in rabies infection are needed. However it appears that they also contain viral antigen at some stages of their development.

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SOME PROBLEMS OF ELECTRON MICROSCOPY IN THE STUDY OF VIRUS-INFECTED CELLS*

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In the electron microscopy of virus infected cells, the problems are different from those found in the study of purified preparations of virus because the infectivity of particles visualized in cells cannot be tested directly by inoculation of animals or tissue cultures. In the absence of direct tests of infectivity, attempts at visualization of viral particles in cells have utilized comparisons of infected with uninfected cells. Under these conditions, however, it is possible that particles other than those of virus may appear as the result of viral action and may be mistaken for viral particles. For these reasons, we prefer to speak of viruslike particles, although it seems fairly sure in some instances that viral particles have been identified in cells. The effort to recognize viral particles in cells seems worth while, in spite of its difficulties and pitfalls, because of the insight that it may give into host parasite relationships. This paper is not intended as a review of the literature, but will point out problems that have arisen from our own work and exemplify some of the problems that must be dealt with in the electron microscopy of almost any kind of virus infected cells.

Our first electron microscopic observations were carried out with the lungs of mice infected with influenza virus, since earlier studies by light microscopy had shown numerous cytoplasmic inclusion bodies† in cells of the bronchial epithelium.[1] The most pertinent facts from our early and recent studies by light microscopy may be summarized as follows. The inclusions were present after infection with several strains of Type A influenza virus with Type B and with strains of virus that grow in the lung without producing pneumonic consolidation. Cytoplasmic inclusions were not present in the bronchial epithelium of uninfected mice, mice infected with pneumonia virus of mice (PVM) or mice with pulmonary consolidation from Newcastle disease virus (NDV). Most of the inclusions were deeply basophilic but acidophilic inclusions were present also. Nuclei of the epithelial cells did not show stages of pyknosis, and specific silver stains for cilia indicated that ciliated cells were not destroyed by the infection. Observation of moving cilia in wet preparations confirmed the persistence of ciliated cells. Use of the method of fluorescent antibody was not successful because the inclusions showed autofluorescence without exposure to antibody (FIGURE 1). Cells of uninfected bronchial epithelium did not phagocytize bacteria, and injection of bacteria into lungs infected with influenza virus did not show that the viral infection had [illegible]helial cells capable of phagocytosis. Additional evidence [illegible]

* These investigations were carried out under the sponso[illegible] Respiratory Diseases, Armed Forces Epidemiological B[illegible] The Surgeon General, Department of the Army.

† In speaking of inclusion bodies, we mean bodies [illegible] concerning their causation or composition.

that the inclusions might consist of phagocytized nuclear debris was the finding that numerous inclusions developed during infection of mice that had received roentgen radiation of such a dose as to eliminate leukocytes from the viral lesion of the lung. Most of the inclusions gave a positive Feulgen reaction[2] (FIGURE 2), and the Feulgen raction could be prevented by prior treatment[3] of sections with desoxyribonuclease.*

Electron microscopy confirmed the presence of inclusion bodies in the cytoplasm and failed to show lesions of the nuclei. Sections of cells frequently *permitted visualization of the nucleus with one or more inclusion bodies in the* cytoplasm of the same cell. The inclusions contained particles that were estimated to be in the size range of influenza virus, and the suggestion was made



[illegible] At the same time it was [illegible] ets of endo [illegible]

Subsequent electron microscopy of cells infected with adenoviruses showed [illegible] th [illegible]

form in size. The size of the particles in adenovirus infected [illegible] measured, and was only slightly less than that determined by other workers with other methods. In addition, the particles often occurred in crystal like arrangement and were shown to have internal structure resembling that of large viruses.[5]

This experience with viruslike particles in cells infected with adenoviruses

* Obtained from the Worthington Biochemical Co., Freehold, N. J.

led us to expect intracellular viruslike particles to be smoother, more discrete, and more uniform than the particles we had visualized in the cytoplasmic inclusions of epithelial cells infected with influenza virus In addition, it was reported by Morgan *et al*[6] that viruslike particles could be recognized on the cellular surfaces of chorioallantoic membranes of embryonated eggs infected with influenza virus These workers also examined the lungs of mice infected

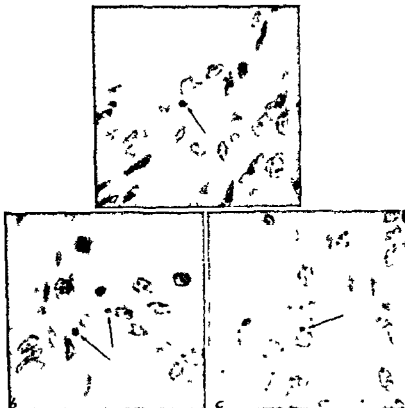


Figure 2 Bronchial epithelium of mice 40 hours after exposure by aerosol to the Weiss strain of Type A influenza virus Feulgen reaction without counterstain Arrows indicate Feulgen positive cytoplasmic inclusion bodies ×9233

with influenza virus and thought that the cytoplasmic bodies were not composed of viral particles because the particles lacked uniformity and internal structure They also found bodies with a similar appearance in chick embryo membranes infected with vaccinia fowlpox and herpes viruses A further reason for doubting that particles in the influenzal inclusions were particles of influenza virus arose from the findings of a number of workers concerning the nucleic acid present in purified preparations of influenza virus obtained from allanto

fluid Although virus from allantoic fluid may not necessarily have the same composition as that purified from mouse lung, it is noteworthy that highly purified preparations of influenza virus from allantoic fluid contain nucleic acid chiefly in the form of ribonucleic acid (RNA) and only trace amounts of desoxyribonucleic acid (DNA) or none at all[7, 8] Since the inclusions were primarily composed of particles giving a positive Feulgen reaction and since this reaction was prevented by prior treatment with desoxyribonuclease, it seemed probable that they contained large amounts of DNA and were unlikely to be viral particles Our present tentative view, therefore, is that the cytoplasmic inclusion bodies induced by influenza virus in our experiments consist, to a large extent, of DNA accumulating in the cytoplasm as the result of viral infection *

Estimation of the size of viruslike particles in infected HeLa cells had some value favoring their identification as adenoviruses, and we used polystyrene latex spheres as internal standards of measurement[10, 11] In this method the spheres of known size are sprayed on the surface of sections, and have the ad

... others using
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may be mentioned that fixation with osmium tetroxide causes some shrinkage of tissue and that sections through various levels of viruslike particles result in images that are usually smaller than the original particles because they do not go through the center It is also known that latex spheres expand slightly from exposure to the beam of electrons[12]

The arrangement of particles in regular rows served as a distinguishing feature of the viruslike particles observed in the nuclei of HeLa cells infected with adenoviruses,[5] Morgan *et al*[14] have shown that very large crystalline aggregates of viruslike particles occur Such large crystals can be visualized by light microscopy[15] and sections adjacent to those examined by electron microscopy have shown that the crystals give a positive Feulgen reaction[16] Since the viruslike particles are closely packed in the crystals, this evidence is an indication that adenovirus contains DNA In this connection, it may be pointed out that nuclei of HeLa cells infected with Type 5 adenovirus contain ... smaller than viruslike
eaction[17]
ed cells, it would be
desirable to show that the particle itself had unequivocally distinguishing morphologic characteristics The demonstration of internal structure in particles visualized in the nuclei of HeLa cells infected with adenoviruses has shown that they do have structure of considerable distinctiveness[5] and the inner bodies surrounded by membranes resemble particles in cells infected with large viruses Recently, we have sought to visualize the internal structure of adenoviruslike particles more clearly and have employed the method of Watson[18]

* Sellers[9] was unable to show greater than normal amounts of *thymidylic acid* in the lungs of mice infected with influenza virus



FIGURE 3 Portion of the nucleus of a HeLa cell 42 hours after infection with Type 3 adenovirus Electron micrograph shows internal structure of viruslike particles The section was stained with uranyl acetate and covered with collodion ×130,000

in which the sections are stained with uranium acetate and covered with an additional layer of collodion in order to increase contrast FIGURES 3 and 4 show examples of the findings and it may be seen that many particles have a dense inner body surrounded by a membrane Other particles consist essentially of two concentric circles occasionally the inner circle contains a small

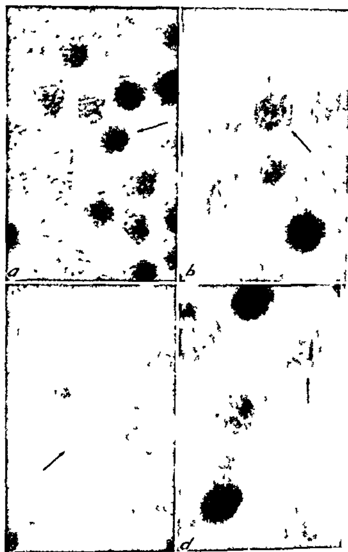


FIGURE 4
Type 3 aden
membrane
membranes
×160 000

granule While these different forms may have resulted from cuts at different levels in the particles, it seems more likely that different forms of the particles exist

It was pointed out that present methods do not permit direct infectivity tests of particles in sectioned cells so that the true viral nature of intracellular particles cannot be tested Nevertheless, it has been shown[19, 20] that purified

pellets of avian leukosis viruses contained particles with internal structure resembling closely the viruslike particles seen in sectioned cells. This method of comparing the structure of particles that can be tested for infectivity with particles visualized in sections seems to offer an approach to the problem as



Figure 5. Electron micrograph of HeLa cell 21 hours after infection with Type 2 poliovirus. There are fine granules within the distorted nucleus and in localized areas outside of the nuclear membrane. X45,000

to how to ascertain the infectivity of intracellular particles. However, in recently reported sections of pellets of purified poliovirus, no membrane was found to surround the particles.[2] It is not clear at present whether the lack of a membrane was the result of difficulties in resolution of this very small viral particle or whether the membrane does not exist.

In recent studies of HeLa cells infected with poliovirus we have employed a Type 2 strain both before and after adaptation in HeLa cells by serial passage. Sections were prepared as in previous studies[4] and also by the method of Watson[5] noted above FIGURES 5 and 6 show distorted nuclear membranes



FIGURE 6 Electron micrograph of HeLa cell 21 hours after infection with Type 2 poliovirus Above the distorted nucleus is an area containing fine granules similar to those present inside the nucleus ×6322

and many small particles or granules within the nucleus and in large extranuclear clusters Examination of such particles under high magnification not only showed no internal structure but failed to show smooth margins or regular shape This experience is similar to that of Kallman *et al*[2] who found no viruslike particles in tissue cultures of monkey kidney cells infected with polio-

virus These investigators also calculated from titrations of the culture fluids that too few viral particles were present to be identified by electron microscopy It is known moreover, that poliovirus is released rapidly from cells in tissue culture,[22, 23] so that the number of viral particles remaining in the cells might be expected to be minimal in number Conversely, adenoviruses are not easily released from cells,[24] and viruslike particles are readily found in cells of in

cell system under study

The failure to find viruslike particles in cells undergoing a severe cytopathogenic effect from infection with poliovirus is consistent with the view of Ackermann *et al*[25] that cytopathogenicity is at least partially a function of the toxic effect of the virus A similar view arose from our finding that viruslike particles were found in only a minority of HeLa cells inoculated with adenovirus

place If a large part of the cytopathogenicity of some viruses in tissue culture is the result of toxic action as appears to be the case in these examples, it may be worthwhile to speculate concerning possible cell virus systems in which a cytopathogenic effect would not take place because the virus lacked toxicity for the particular cells employed, but might be visualized inside of cells by electron microscopy

Finally, the problem of artifacts in the electron microscopic study of lesions in cells induced by viral infection must be considered Moreover, variations from preparation to preparation may make it difficult to compare infected and uninfected cells In HeLa cells the cytoplasm usually shows structures roughly the size of mitochondria however, these do not have the typical cristae usually needed for the identification of mitochondria Some of such structures probably consist of mitochondria that have undergone degenerative changes as the result of some premortem influence such as viral infection or have this appearance because of changes that have taken place during preparation of the cells for electron microscopy[27] In addition, we think that many of such structures are the inclusion vacuoles resulting from pinocytosis, since uninfected HeLa cells grown in media containing colloidal gold show particles of gold in globoid structures of the cytoplasm but not in typical mitochondria.[28] We have found also that similar ingestion of colloidal gold takes place in HeLa cells undergoing cytopathogenic effect from the action of adenovirus

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RELATION OF VIRUSES TO INCLUSION BODIES

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In this paper, I shall discuss first the basic reaction patterns of cells to viral infections as they are observed under the conventional light microscope, and attempt to correlate these patterns with some of the data obtained by the use of newer techniques. Second, I shall review certain observations on the intracellular multiplication of rickettsiae which may be pertinent to the analogous problem of the cytoplasmic and nuclear multiplication of viruses.

History

The molluscum body was described in 1841 and the cytoplasmic inclusion in fowlpox (the Bollinger body) in 1873. These bodies, together with other similar structures described later, were long believed to be protozoa or fungi. Borrel[1] in 1904, showed that the Bollinger body was composed of myriads of minute coccoid elements, each about 0.2 μ in diameter. In 1929 Woodruff and Goodpasture[2] showed that isolated washed Bollinger bodies were infective.

The nuclear inclusions of herpes simplex were described by Lipschutz in 1921. George Hass and I, in 1932, observed compact nuclear clusters of spotted fever rickettsiae in tissue cultures and stressed the resemblance of these intranuclear aggregates to the nuclear inclusions seen in certain viral infections.[3]

Thus the general concept that certain viral inclusions, both cytoplasmic and nuclear, might be colonies of viral particles gradually gained momentum, and in the case of several of the larger viruses was confirmed by direct observation, although the occurrence of a matrix in which the elementary bodies were embedded somewhat complicated the picture.[4] The nature of the homogeneous inclusions associated with the smaller viruses remained in the realm of speculation and has only recently been partially clarified by the use of the electron microscope.

Meanwhile, new virological concepts were being formulated and developed in the general areas of the host-parasite relationship and in the method of multiplication, notably the diversion of enzymes of their host cells to viral replication, the multiplication of the smaller viruses by a mysterious method differing from that of binary fission, the breakdown of virus particles into subunits that were reduplicated separately and later combined to form mature

. . . .

the important questions obviously are what enzymes are diverted and how and in what direction they are diverted. By what type of biochemical persuasion does the virus particle, after entering its host cell, divert its enzymatic machinery to its own replication? At present we possess several new techniques for asking specific questions in this field of inquiry, and we are getting

answers to these questions To be sure, each answer raises several new ques

Basic Reaction Patterns of Cells to Viral Infection

Since all cytopathic reactions of cells to viral infection are interrelated, it seems desirable to discuss them as a whole before considering the special case of inclusion body formation Cellular alterations may be observed in undisturbed animal tissues or in cells isolated in tissue culture The latter method has the advantage of eliminating those modifications of the reaction patterns resulting from such factors as antibody formation and nonspecific inflammation Certain specific alterations, however, notably neoplasia and hypoplasia, can be observed only in the intact animal

Under the light microscope, virus infected cells may appear unaltered, or may show (1) staining alterations, (2) assumption of spherical shape, (3) necrosis, (4) hypertrophy, (5) giant cell formation, (6) hypoplasia, (7) metaplasia, (8) hyperplasia, (9) neoplasia, or (10) inclusion body formation The various alterations may be observed in a wide variety of combinations that may occur simultaneously or sequentially The formation of syncytial giant cells and inclusion bodies, for example, may appear to be simultaneous, but staining alterations, hyperplasia (proliferation), inclusion body formation, and necrosis often occur sequentially Sequential changes are best studied in tissue cultures, but the various developmental stages often may be observed in infected animal tissues and correlated with observations made in tissue cultures There are some obvious advantages in studying intact cells in living animals, since cells in tissue culture are under highly artificial conditions It is, in fact, rather surprising that there is, in general, excellent correlation between observations made under such different conditions The principal difference is that latently infected animal tissues without inclusions or necrosis may develop inclusions and necrosis when grown in tissue culture, or when tissue cultures are inoculated with their cell free extracts

The absence of cytological change in virus infected cells is readily explained by assuming that relatively few virus particles are uniformly distributed in

testicular tissue from one rabbit into the testis of another, ...

injury is indicated by the occurrence of nuclear inclusions and necrosis

Staining alterations alone usually represent an early stage in development

formation is particularly prominent in measles, varicella, and canine distemper, but it should be noted that this process in the living animal is seen only in certain organs and in certain developmental stages of the infection Hypoplasia (arrested embryonic development) is exemplified by the cerebral lesions

viral diseases Hyperplasia may be diffuse and of moderate degree (the neuroglial cell proliferation in chronic inclusion encephalitis) or localized and extensive, as in molluscum contagiosum or infectious rabbit papillomatosis Malignant neoplasia initiated by viral infection has been found only in lower animals

Factors Influencing the Reaction Pattern

Duran Reynals[7] has shown that the Rous sarcoma virus may cause necrosis in young chickens while older chickens react to the virus by developing malignant neoplasia Similarly, the reaction pattern may be influenced by genetic constitution (either of the virus or of the host), hormones antimetabolites and by a variety of enzymatic inhibitors and activators Of particular interest are [illegible] and compounds [illegible] example) Pre- [illegible] th the formation of virus nucleoproteins, but in no instance has the precise mechanism been determined

Inclusion Body Patterns in Viral Infections

The visualization of relatively minute aggregates of elementary bodies by the electron microscope probably makes it necessary to broaden our definition of the term inclusion body Conventional inclusion bodies, however, as seen under the light microscope, range in diameter from 1 or 2 to 20 or more µ in greatest diameter They may be found in association with any of the other cytological alterations described above including in lower animals neoplasia

In about one half of the known viral infections of man and lower animals, inclusion bodies have not been observed As suggested above, this is probably because there are no visible aggregates of elementary bodies or of their component parts Inclusion bodies may be present in cells without other cytopathic changes but this probably represents early infection, necrosis or atrophy presumably follows, although the possibility of eventual recovery has not been excluded

Inclusions may occur in the nucleus, in the cytoplasm, or in both On the basis of the colony formation theory, one would be tempted to associate these three patterns with preferential multiplication in the nucleus or cytoplasm because of different metabolic requirements Observations with the light microscope do not justify this hypothesis, however, since, at least in the case of small viruses a cell showing only a nuclear inclusion might contain large numbers of uniformly distributed virus particles in its cytoplasm and the reverse situation also might result

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The absence of cytological change in virus infected cells is readily explained by assuming that relatively few virus particles are uniformly distributed in cytoplasm or nucleoplasm An example of this situation is found in virus III infection in the testis of the rabbit, no cellular alterations are seen unless the degree of infection is magnified by the artificial method of injecting homogenized testicular tissue from one rabbit into the testis of another, whereupon cellular injury is indicated by the occurrence of nuclear inclusions and necrosis

Staining alterations alone usually represent an early stage in development

fected cells (not merely their inclusions) were thought to [illegible] protozoa[5] until Goodpasture,[6] in 1921, published evidence that these peculiar cells were derived from normal cells by a type of cytomorphosis Giant cell

formation is particularly prominent in measles, varicella, and canine distemper, but it should be noted that this process in the living animal is seen only in certain organs and in certain developmental stages of the infection Hypoplasia (arrested embryonic development) is exemplified by the cerebral lesions resulting from intrauterine infection of infants with the salivary gland virus Squamous metaplasia is prominent in the bladder of minks or ferrets suffering from distemper and in pulmonary alveolar duct epithelium in a number of viral diseases Hyperplasia may be diffuse and of moderate degree (the neuroglial cell proliferation in chronic inclusion encephalitis) or localized and extensive as in molluscum contagiosum or infectious rabbit papillomatosis Malignant neoplasia initiated by viral infection has been found only in lower animals

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Certain specific cytological changes are associated with inclusion body formation. In the case of cytoplasmic inclusions, the peripheral clear zone or halo is most prominent. With *Type A nuclear inclusion* formation, the nuclear chromatin may be marginated, often with two distinct marginal polar bodies, chromatin may disappear from the body of the nucleus, so that it appears empty except for the inclusion. Wrinkling of the nuclear membrane also is common.

In certain viral infections associated with both nuclear and cytoplasmic inclusions, the cytoplasmic bodies are stained brightly by the periodic acid Schiff (PAS) method, while the nuclear inclusions remain unstained, cytoplasmic inclusions usually remain unstained by silver methods, while nuclear inclusions are deeply stained. These reactions perhaps may be correlated with the cytoplasmic multiplication of the polysaccharide protein unit of the virus particle and with the replication of the nucleic acid protein portion in the nucleus, which has been demonstrated in certain viral infections. In salivary gland virus infected cells, localized areas of fine dustlike PAS positive material are often noted in the cytoplasm.

Electron microscopic studies of viral inclusions have, in general, confirmed the colony formation theory, in that most inclusions that have been studied appear to contain elementary bodies at some stage in their development. Homogeneous bodies may appear first (perhaps representing the accumulation of macromolecular building blocks), and the virus particles, or their subunits may separate later from this matrix. In other instances, the elementary bodies multiply first, and the mature inclusion seems to be a product of their degeneration. Further studies are needed, however, since sequences are difficult to establish in killed tissue. Cinematographic studies of living cells under the electron microscope would be ideal, but this method of approach is not yet available.

Rickettsial Multiplication in Cytoplasm and Nucleus

Rickettsiae differ from the smaller and more typical viruses in their bacteriumlike morphology, their multiplication by binary fission, and their sensitivity to certain antibiotics. The large viruses of the lymphogranuloma psittacosis group, however, resemble the rickettsiae in the above respects. Rickettsiae, like viruses, are obligate intracellular parasites, and separation of the two groups must be made on rather arbitrary grounds.[10]

Cells infected with rickettsiae show patterns similar to those seen in virus infected cells: diffuse cytoplasmic growth (rickettsiae of the typhus group), cytoplasmic clusters (*Rickettsia burneti*), and intranuclear aggregates (rickettsiae of the spotted fever group). The latter show their intranuclear growth best in tissue cultures and in the tissues of their arthropod hosts (ticks). In addition to their massive intranuclear growth in tissue cultures, spotted fever rickettsiae also grow sparsely in the cytoplasm. Typhus rickettsiae are never seen in nuclei and neither typhus nor spotted fever rickettsiae are ever found in nucleoli. The latter structures remain clear even when nuclei are packed and even distended with spotted fever rickettsiae.

The antibiotic response patterns of typhus and spotted fever rickettsiae are identical. In guinea pigs, sulfa drugs increase rickettsial growth; in the case of spotted fever rickettsiae, the increased growth occurs particularly within

nuclei In fertile eggs penicillin, chloramphenicol, streptomycin and the tetracycline drugs all cause marked inhibition and almost complete cessation of the multiplication of both types of rickettsiae Para aminobenzoic acid and high incubation temperature (40° C) are rickettsiostatic for both types of rickettsiae Cells massively infected with typhus rickettsiae are distended to two or three times their normal diameter and assume a spherical shape Nuclei heavily infected with spotted fever rickettsiae usually retain their normal shape but frequently their diameters are doubled and there is definite margination of the nuclear chromatin

We have found only one enzymatic modifier that shows antithetical effects on rickettsiae of the two groups Radiation which in the fertile egg markedly enhances the growth of *Rickettsia mooseri*[11] (a member of the typhus group) inhibits and under certain conditions completely supresses the multiplication of *Rickettsia akari*[12] (a member of the spotted fever group) The rickettsiostatic action on *R akari* is noted when single doses of X radiation are given but is most striking and complete when tritiated water a source of continuous beta emission is injected into the yolk sac Both the increased growth of *R mooseri* and the inhibition of *R akari* are roughly proportional to the dosages of radiation used

It seems probable that these antithetical reactions to radiation may reflect a metabolic difference between the two organisms which is correlated with their different cytological growth patterns The important effects of radiation on nucleic acid metabolism would afford a logical explanation in general terms of this correlation Radiation is known to exert a number of different effects on cellular metabolism including the inhibition of nucleic acid synthesis conversion of ribonucleic acid (RNA) to desoxyribonucleic acid (DNA) inhibition of oxidative phosphorylation depolymerization of nucleic acids and inhibition of sulfhydryl enzymes in general It could be expected therefore that any particular biological effect observed would depend on the resultant of these various activities In the case of enhancement or stimulation of rickettsial growth in fertile eggs however the almost quantitative effects of various dosages over a wide range compatible with continued embryonic development encourage us to believe that some one specific radiation effect may play the important and dominant role *By using agents that exert separately the various effects of radiation it may be possible to pinpoint this specific effect*

In studying the effects of X radiation on the multiplication of R mooseri in fertile eggs two incidental observations were made First the rickettsiostatic effect of high incubation temperature (40° C) was reversed by radiation in irradiated eggs rickettsiae grew just as freely at 40° C as they did at 37° C without radiation[13] Second the rickettsiostatic action of streptomycin was reversed by X radiation and this reversal was quantitative in nature with increasing doses of streptomycin larger doses of radiation were required to inhibit *the rickettsiostatic action*[14] *Similar studies on Rickettsia akari have not* yet been carried out

Effects of Radiation on Viral Multiplication

We have studied the effect of radiation on only one virus the PR8 strain of influenza A virus[15] Groups of embryonate eggs were given 250 500 750 and

1000 r of X radiation. Eighteen hours later, these 4 groups, together with a control group, were injected intraallantoically with the virus. Radiation at all dosage levels caused significant changes in the infectivity titer curves during the next 90 hours. The most striking effect noted, however, was the slow development and very low level of the infectivity titer that followed the administration of 750 r per egg; other dosage levels resulted in less obvious effects. It seems probable that in these experiments two or more antagonistic effects of radiation may be involved, although it also is possible that radiation may have exerted different effects on the reduplication of the viral subunits.

It would seem desirable to study the effects of radiation on a number of other viruses, selected on the basis of their multiplication in the cytoplasm, in the nucleus, or in both parts of the cell. Insofar as I am aware, studies of this type have not been carried out in a systematic manner.

Relation of Nucleic Acid Composition to Viral and Rickettsial Multiplication

Analysis of typhus rickettsiae indicates that they contain both DNA and RNA, the DNA predominating. Unfortunately, I have been unable to find reports on the nucleic acid makeup of rickettsiae of the spotted fever group. This information might furnish an important clue to the preferential localization of the two types of rickettsiae in the cytoplasm or nucleus, respectively, and also to their antithetical response to radiation.

The nucleic acid composition of a number of purified viruses has been determined. Since the outstanding difference between nucleoplasm and cytoplasm is the preponderance of DNA in the former and the almost exclusive presence of RNA (under normal conditions) in the latter, it seems possible that the preferential multiplication of virus particles (or of rickettsiae) in one or the other part of the cell may be related to this basic difference. If it were true that RNA viruses always multiplied in the cytoplasm and DNA viruses in the nucleus, the problem would be greatly simplified. Since the simple relationship obviously does not hold, some other type of relationship must be sought.

The development of DNA viruses in the cytoplasm where little or no DNA is normally present makes it clear that such viruses are able to cause profound alterations in the nucleic acid metabolism of their host cells. In the case of the bacteriophage the evidence is against the direct transfer of bacterial nucleic acid to the viral particles, although a certain proportion of bacterial DNA is available after partial breakdown for the formation of viral DNA.[18] Presumably, therefore, most of the viral DNA must be constructed from smaller building blocks available in the surrounding medium. The rapidity with which *some DNA viruses multiply* in the cytoplasm makes one wonder what happens to the RNA normally present there. Possibly it is broken down to nucleotides, or smaller units, which are then used in building DNA according to the specifications of the newly arrived virus particle. Thus some viruses, either by virtue of an intrinsic enzymatic property, by diversion of enzymes in their host cells, or by evoking or unmasking latent host cell enzymes, may be considered to act as nucleic acid converters (RNA to DNA) when they replicate in host cell cytoplasm.

In the study of such phenomena, one is apt to look on the living molecules of the viral components as the keys to the situation. A consideration of cells

lar machinery as are their smaller and more glamorous relatives. Possibly, rickettsial multiplication may be explained on some relatively simple basis such as the segregation of nucleic acid by its incorporation in their bodies with consequent disturbance of some normal equilibrating mechanism and the initiation of a chain reaction In the present state of our knowledge one scarcely needs to apologize for the introduction of highly speculative suggestions In any case, the intracellular multiplication of rickettsiae may involve in a simplified form many of the problems concerned in viral multiplication and it therefore seems worthy of further study by the newer methods of analysis

Conclusion

The nature of viral inclusions is part of a more general problem the kinetics and biochemistry of those mysterious processes by which viruses and rickettsiae multiply within susceptible cells This problem in turn is related to the cytology and physiology of cells under normal and pathological conditions Even though we have at present only incomplete and often apparently conflicting fragments of information in these areas the use of new techniques of study may be expected to result in the near future in a greater degree of order and harmony The almost incredible progress made in recent years certainly justifies this assumption and there is every reason to believe that this progress will continue at an accelerated rate not only by the accumulation of more data but by the correlation and synthesis of data already in our possession

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